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Effect of ionization technology on microbial contamination and food quality in food storage equipment

in collaboration with Electrolux Italia S.p.A.

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Table fo contents

ABBREVIATIONS

DA	device A
DB	device B
DM	device M
ESCS	electrostatic space charge system
NAI	negative air ions
O3	ozone
PAI	positive air ions
refT	refrigeration temperature
RH	relative humidity
roomT	room temperature
ROS	reactive oxygen species
VOCs	volatile organic compounds

PREFACE

The research in the food industry is constantly looking for innovative technologies able to make food safe by maintaining its fresh-like characteristics and, at the same time, be also green and sustainable. This need arises from the consumers who have developed a new concept of food, thinking of it as a safe, healthy, and high-quality food having fresh-like characteristics. Trying to meet these requirements, special attention has been paid to non-thermal technologies that, by not generating high temperatures, can better maintain the nutritional components and the sensory properties of the food. However, most of the technological interventions took place in the food processing plants, and once in the consumer's homes, the fresh foods are subjected to decay. To supply this lack, some strategies have been approached by the food industry, like fridge surfaces containing silver, photocatalytic filters, or ionization modules.

Based on these considerations, this Ph.D. thesis aimed to investigate the effect of ionization technology for improving the microbiological quality of food storage equipment and to evaluate its effect on the food appearance during storage.

SUMMARY

In this Ph.D. thesis, ionization technology has been studied from different points of view to investigate the possibility of using it inside domestic refrigerators to control microbial contamination during refrigerated storage without affecting the quality of the foods. Different concentrations of NAI and ozone were evaluated on food-related microorganisms belonging to different species: Gram-positive and Gram-negative bacteria, yeasts, and molds. Ionization technology was effective in reducing the microbial counts of the selected species, achieving significant results for some peculiar species of interest in the food industry, like the pathogen *Listeria monocytogenes* and the typical food spoilage bacterium *Pseudomonas fluorescens*. These promising results have led to the possibility to use this technology during refrigerated storage for improving the hygienic status of the environment in which the food is stored. Therefore, the study of the effect of the same concentrations of NAI and ozone was carried out on fruits, vegetables, and mushrooms, showing that a high concentration of ozone was a critical factor since it caused oxidation phenomena on foods and it did not meet the requirements for a safety and security exposition. Based on these findings, the use of NAI and low ozone concentration was investigated in a simulated real-world application inside a domestic refrigerator. The antimicrobial effect has been studied on different materials used for the construction of internal refrigerator lines. Then, the effect was examined on lettuce, strawberries, and mushroom through a color vision system and physical analysis, showing that the use of NAI under low concentration of ozone can improve the microbiological quality of the surrounding environment without affecting the quality of the tested foods.

1 INTRODUCTION

1.1 Non-thermal technologies: a new way for food safety and quality

Nowadays, the research in the food industry is constantly looking for new technologies able to make food safe by maintaining its fresh-like characteristics and, at the same time, to be also green and sustainable. Or technologies already known but applied in such a new way as to make them innovative. This need arises since the concept of food preservation has evolved over the years. The traditional thermal technologies, such as pasteurization and sterilization always allowed to guarantee the safety and stability of the foods, especially from the microbiological point of view. But they can negatively affect some food ingredients, particularly heat-sensitive compounds, like vitamins, enzymes, or polyphenols that are strictly related to the quality and healthy aspects of the food (Morales-de la Peña et al., 2019). In recent years, consumers have developed a new concept of food, thinking of it as a safe, healthy, and high-quality food having fresh-like characteristics. For that reason, special attention has been paid to non-thermal technologies that, by not generating high temperatures, can better maintain the nutritional components and the sensory properties of the food, guaranteeing at the same its safety. Some examples of such technologies are pulsed UV-light, ultrasound, irradiation, cold plasma, pulsed electric fields, and high hydrostatic pressure (Zhang et al., 2019). Many authors have studied the antimicrobial potential of these technologies demonstrating their capacity to reduce the microbial load without negatively affecting the quality of the food (Holck et al., 2018; Khan et al., 2019; Shalaby et al., 2016; Ziuzina et al., 2016). In the wide scenario of the non-thermal technologies, the

atmospheric cold plasma (NTP) has sparked particular interest, since it can be considered also an oxidative technology, the principal characteristic that makes it similar to the ionization technology that it is intended to investigate in this thesis project. The antimicrobial effect of cold plasma was proved in vitro (Lee et al., 2006; Scholtz et al., 2010) and directly on food, demonstrating its capacity to reduce the microbial population maintaining the nutritional and quality characteristics of the food (Korachi et al., 2015; Min et al., 2017; Misra et al., 2014; Rossow et al., 2018). NTP has been described by López et al. (2019) as "a promising technology for food preservation and maintenance of food safety, with minimal impact on the quality attributes of foods, thanks to its effectiveness in microbial inactivation, including of pathogens, spoilage fungi, and bacterial spores, simple design, ease of use, cost-effective operation, short treatments times, and lack of toxic effect". From this description, very important characteristics can be deduced, as the ease of use and simple design that makes it useful also for an inexpert operator, the economical aspect that makes it available for all the consumers without increasing the production costs, and the absence of toxic substances that make it safe and sustainable for the use, the food, and the environment. Considering the similarity with NTP with ionization technology and, therefore, this series of advantages, an in-depth study of ionization technology has been done.

1.2 Ionization technology

1.2.1 Air ions: generation and composition

Negative air ions were discovered at the end of the 19th century in Germany and England (Krueger & Reed, 1976). From their discovery, as extensively reviewed by Jiang et al. (2018), the biological effects on humans, animals, plants, and microorganisms have been widely studied, and the interest remains high also nowadays. The term air ions refers generally to all airborne particles that possess electrical charges whose movements are influenced by electric fields (Tammet, 1997). Neutral molecules in the air can become naturally ionized following the accumulation of enough energy from radiation, ultraviolet light, cosmic radiation, atomization of water, movement of large masses of air, and lighting (Krueger, 1969). In addition to the natural source, air ions can also be formed artificially by photon, and nuclear or electronic ionization processes. Photon ionization displaces electrons from molecules thanks to a low-energy

X-ray source; nuclear ionization displaces electrons from molecules through their collision with alpha particles emitted from polonium-210. In both cases, neutral molecules capture electrons displaced and become negative ions (NAI) (Daniels, 2002). Electronic ionization concerns the principle of corona discharge (Figure 1): a sufficiently high voltage is applied between the ground and discharge electrodes to generate an electric field that interacts with molecules producing ions of the same polarity as the applied voltage (Roth, 1995). However, the displacing of electrons leads to the formation of positive air ions (PAI), but it has been shown that, during the electric discharge, negative ions are generated in a higher number than positive ions and that the enormous amount of NAI neutralizes PAI (Y. S. Kim et al., 2011; Wu et al., 2006).

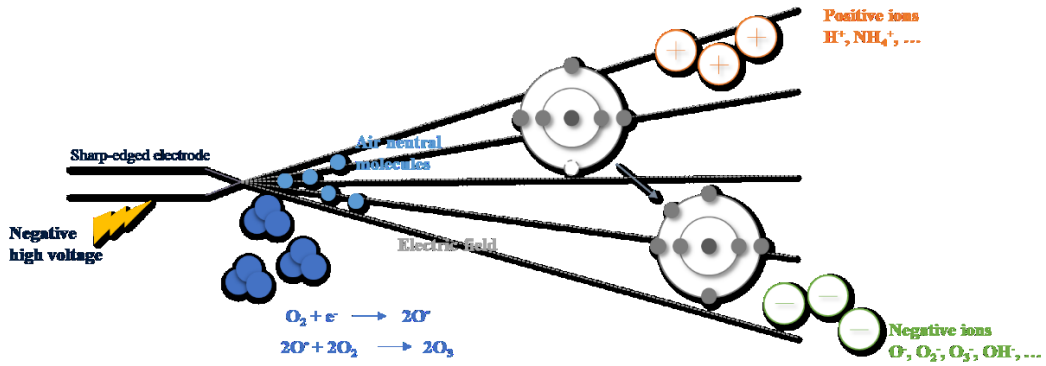

Figure 1 Process generated with a corona discharge method: negative ions, positive ions, and ozone are produced.

Nagato et al. (2006) have carried out a mass spectrometric measurement to observe the evolution of negative air ions produced during a negative corona ionizer applying a discharge voltage of −4.5 kV with a discharge current of 8.5–10.5 µA. They found that the primary negative ions generated in a corona discharge are O^-, O_2^-, and OH^-. Then, they react with components in the air to generate secondary NAI (Skalny et al., 2004). The superoxide ion (O_2^-) represents approximately 95% of the negatively charged ions and is more stable than the other primary ions (Luts & Salm, 1994). O^- and O_2^- react with NO_2, already present in normal air, forming NO_2^- that can be converted to NO_3^- by a reaction with O_3. The O^- and O_2^- ions react also with O_3 to produce O_3^- and the reaction of this one with NO_2 leads to the formation of NO_3^- ions. These ions can be generated also by the reaction of NO_2 with CO_3^-, which is the result of the reaction of CO_2 with O^- and O_3^-. The formation of OH and OH^- ions is probably due to the reaction between O^- and H_2O. Then, OH^- can react with CO_2 to form HCO_3^- (Nagato et

al., 2006). In Table 1 a summary of the above-described reactions with their relative reaction rate constants *k* is reported. The composition of negative air ions depends on the operating conditions during their generation. Wu et al. (2006) have conducted a study to evaluate the effect of relative humidity and distance from the discharge electrode on the concentration gradient of NAI. During the experiment, NAI were continuously generated by electric discharge and detected at 10, 20, 30, 70, 130, 210, 260, 360, 420, 450, 560, 610, 800, and 900 cm space locations, and relative humidity and temperature were between 38.1 and 73.6% and 25.2 ± 1.4 °C, respectively. As regards the behaviour considering relative humidity, results indicated that in the zone near the electrode (10 – 30 cm), the NAI concentration remained almost stable in the relative humidity range tested; in the space between 70 and 360 cm; the concentration of NAI decreased with the increase of relative humidity; in the space location from 420 to 450 cm, the concentration of ions was stable as near the electrode; in the farthest zone (560 – 900 cm), NAI concentration increased when relative humidity increased. Moreover, as regards the distance from the electrode, results showed that the concentration of NAI follows a logarithmic linear tendency within a specific distance depending on the relative humidity values. Luts et al. (2011) have studied the composition of NAI as a function of ion age, relative humidity, and presence of iodine and diethylamine in high concentrations, finding that all these parameters influenced the NAI composition compared to the background air.

Usually, the generation of air ions occurs electrically through the ionizers according to the principle of corona discharge. But this mechanism is also the common one for the generation of gaseous ozone. Indeed, when the oxygen molecule, naturally present in the air, passes through an electrical field splits into two atomic oxygen radicals that combine with the oxygen molecule generating ozone (Guzel-Seydim et al., 2004), according to the following reactions (Tapp & Rice, 2012)

$O_2 + e^- \rightarrow 2O^\bullet$

$2O^\bullet + 2O_2 \rightarrow 2O_3$

Effectively, the presence of ozone is reported in most cases by the authors that have conducted experimentations using ionization technology (L. Fan et al., 2002; Fletcher et al., 2007; Kampmann et al., 2009; Park et al., 2016). Therefore, an overview of ozone and its properties was considered appropriate.

Table 1 Reactions and reaction rate constants (k, $cm^{3/}s$) occurring during corona discharge (from Nagato et al. 2006).

Reactions	k (cm^3/s)
$O^- + NO_2 \rightarrow NO_2^- + O$	1.0×10^{-9}
$O_2^- + NO_2 \rightarrow NO_2^- + O_2$	7.0×10^{-10}
$NO_2^- + O_3 \rightarrow NO_3^- + O_2$	1.2×10^{-10}
$O^- + O_3 \rightarrow O_3^- + O$	8.0×10^{-10}
$O_2^- + O_3 \rightarrow O_3^- + O_2$	6.0×10^{-10}
$O_3^- + NO_2 \rightarrow NO_3^- + O_2$	2.8×10^{-10}
$O^- + H_2O \rightarrow OH^- + OH$	6.0×10^{-13}
$OH^- + CO_2 + M \rightarrow HCO_3^- + M$	7.6×10^{-28}
$OH^- + O_3 \rightarrow O_3^- + OH$	9.0×10^{-10}
$OH^- + NO_2 \rightarrow NO_2^- + OH$	1.1×10^{-9}
$NO_2 + OH + M \rightarrow HNO_3 + M$	3.3×10^{-30}
$O^- + CO_2 + M \rightarrow CO_3^- + M$	3.1×10^{-28}
$O_3^- + CO_2 \rightarrow CO_3^- + O_2$	5.5×10^{-10}
$CO_3^- + NO_2 \rightarrow NO_3^- + CO_2$	2.0×10^{-10}

1.2.2 Ozone: not just a side effect

The ozone has been widely used in the food industry since the end of the 1800s when its disinfectant capacity was discovered. It was used for the sanitization of drinking water (the Netherlands and France), preservation of meat (Germany), sanitization of swimming pools (USA), treatment of shellfish (France), maintenance of a safe environment in egg and cheese storage rooms (USA), treatment of shell eggs to reduce Salmonella (Russia) (Gonçalves, 2009; Tiwari & Rice, 2012). In 1982, in the USA, ozone was declared as GRAS (Generally Recognized as Safe) for the disinfection of bottled water (FDA, 1982), and in 1995 it was used as a sanitizer for process trains bottled water plants (FDA, 1995). In 1997, the Electric Power Research Institute (EPRI) independent expert panel declared ozone as GRAS for direct contact with foods (Graham et al., 1997). In 2001 FDA and Food Safety and Inspection Service (FSIS) of the US Department of Agriculture (USDA) approved gaseous and aqueous ozone as an antimicrobial agent for direct contact with fish, meat, and poultry (FDA, 2001, 2003).

Nowadays, the use of ozone is allowed to replace conventional sanitation methods, but the regulations are different for the countries. In Europe, currently, a specific

regulation for the use of ozone in food processing is missing. Ozone may fall within the definition of novel foods in the Regulation (EU) 2015/2285, which considers belonging to this category also the foods resulting from a production process not used within the Union before 15 May 1997. In some countries (i.e. Italy), ozone treatment is allowed in cheese ripening environments and not on the cheese itself (CNSA, 2010). In Japan, as reported by Naito & Takahara (2006), ozone as a gas or water has been part of food treatment plant processing. In Australia and New Zealand, ozone treatment is considered as a processing aid in the Food Standards Code-Standard 1.3.1, Clause 11 (FSANZ 2013) on food additives: no restrictions are on its use if Good Manufacturing Practice (GMP) is implemented. In the Eurasian Economic Union (EAEU), which consists of Russia, Belarus, Kazakhstan, Armenia, and Kyrgyzstan, there is Technical Regulation TR CU 029/2013 on safety requirements of food additives, flavor enhancers, and processing aid: ozone can be considered as a processing aid in compliance with the requirements specified by the regulation.

Despite this heterogeneity of regulations in different countries, the use of ozone is widely studied for direct use on foods, to improve their microbiological quality and therefore their stability and shelf-life. As underlined in Table 2, good performances of ozone concerning microbial reduction can be achieved. However, the conditions of the treatments must be accurately identified to avoid negative consequences on the treated foods. For example, Liu et al. (2020) treated the mushroom with different ozone gas concentrations (1.07, 2.14, 3.21, 4.28, 5.35, 6.42, 7.49, 8.56, and 9.63 mg/m^3) for 30 min showing that concentrations higher than 3.21 mg/m^3 lead to the browning and injuries to the food. This behavior is due to the high oxidizing potential (2.07 V) (Greene et al., 2012), which gives it an indiscriminate reactivity with components of both organic and inorganic nature, interacting too strongly with food components and causing oxidation phenomena that are not acceptable from a sensory point of view.

Table 2 Some examples of treatments with ozone for the improvement of microbiological quality of food.

Food	Treatment condition	Log reduction	Quality observation	References
Cantaloupe melon juice	7.0 ± 2.4 g/L gaseous ozone, 30 and 60 min	Alicyclobacillus acidoterrestris spores: 0.73 – 2.22	Degradation of color, vitamin C, carotenoids, total antioxidant activity. Increase of total phenolic content.	(Fundo et al., 2018)
Red bell peppers	2 ppm gaseous ozone, 3 min	Listeria innocua: 2.8 ± 0.5	-	(Alexandre et al., 2011)
Strawberry		Total mesophiles: 2.3 ± 0.4		
Watercress		Total coliforms: 1.7 ± 0.4		
Cantaloupe melon peel	31 g /L gaseous ozone, 30 and 60 min	Listeria innocua: 1.2	Loss of antioxidant activity. Increase of color, vitamin C, total phenolic content.	(Miller et al., 2021)
Green leaf lettuce	2 ppm ozonated water, 2 min	Aerobic mesophilic count: 1.5 Psychrotrophic count:1.1 Enterobacteriaceae:1.5	No negative effect on vitamin C, β-carotene. Maintenance of sensory qualities during cold storage.	(Ölmez & Akbas, 2009)
Carrot	0-5 mg/L gaseous ozone 0-10 mg/L ozonated water		No negative influence on weight loss %, color change, firmness, and soluble solids. Variation in pH.	(Souza et al., 2018)
Shiitake mushroom	3.21 mg/m^3 gaseous ozone, 30 min	Total bacterial count: 0.7 Yeast and mold: 1.2	No negative influence on total phenolic compounds, soluble protein content. Increase of antioxidant enzyme activity.	(Liu et al., 2020)
Boneless and skinless turkey breast meat	10 g/m^3 gaseous ozone, 8 h	Total aerobic mesophilic bacteria: >3.0 Yeast and mold: 1.7 Enterobacteriaceae: 2.0	Changes in carbonyl contents, thiobarbituric acid reactive substances, color, and pH. Increase of water holding capacity and cooking yield.	(Ayranci et al., 2020)
Pond-raised tilapia	6 ppm ozonated water, 1 h	Aerobic bacteria: >3.0	Quality improvement during storage; shelf-life extension.	(Gelman et al., 2005)

1.2.3 Chemical and physical properties of ozone

Ozone is an allotrope of oxygen consisting of three oxygen atoms forming an obtuse angle (116°49′) with a central oxygen atom attached to two equidistant oxygen atoms. In the valence shell of each oxygen atom, there are six electrons with a $2s^2\ 2p^4$ electronic configuration. The central atom is linked to one oxygen atom with a double bond (shorter) and the other with a single bond (longer). However, the three atoms are equidistant (bond lengths 1.278 Å), and thus, a rearrangement of the orbitals has occurred with the formation of nine sp^2 hybrid orbitals that move throughout the ozone molecule giving four resonance structures (Figure 2). The oxidizing potential of ozone is 2.07 V, which is high if compared with that of oxygen (1.23 V) (Greene et al., 2012; Oehlschlaeger, 1978).

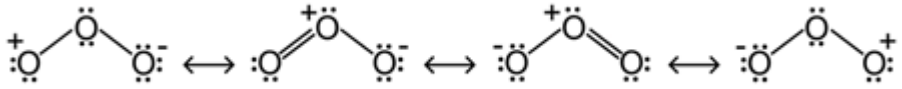

Figure 2 Four resonance structures of ozone molecule (Oehlschlaeger, 1978).

The physical properties of ozone are reported in Table 3.

Table 3 Physical properties of ozone (Greene et al., 2012; Manley & Niegowski, 1967; Rice et al., 1981).

Color	light blue (NTP: 0 °C, 1 atm) dark blue (condensed)
Density	2.1415 g/L (NTP: 0 °C, 1 atm) 1.354 kg/L (condensed)
Boiling point	-111.9 ± 0.3 °C
Melting point	-192.5 ± 0.4 °C
Critical temperature	-12.1 °C
Critical pressure	54.6 atm
Solubility	0.64 L ozone/L water (0 °C) 0.456 L/L (15 °C) 0.112 L/L (40 °C) insoluble in water at 60 °C

An important parameter is considered solubility in water. It depends not only on the temperature, with an increase in decreasing temperature, but also on pH, presence of substances readily oxidized by ozone, and decomposition of ozone in the gas and liquid phases (Khadre et al., 2001). In detail, ozone is more stable at low than high pH values; the stability is the greatest when pH is 5.0 and at higher pH ozone decomposes

(at pH 9.0 no ozone can be found) (J. G. Kim, 1998). Ozone solubility increases when the purity of water increases, then ozone dissolves faster in deionized and distilled water than in tap water, since it may contain organic matter that consumes ozone and minerals which can catalyze ozone decomposition (Staehelin & Hoigne, 1985). Therefore, depending on the water quality and temperature, the half-life of ozone is in the range of seconds to hours (Tzortzakis & Chrysargyris, 2017). In pure aqueous solutions, ozone decay is characterized by an initial phase of fast decrease followed by a second phase in which ozone decreases with first-order kinetic (Marino et al., 2018). Ozone in water decomposes according to a radical chain reaction following initiation, propagation, and termination steps (Table 4). Ozone in the gas phase decomposes slowly because it is thermally unstable up to 250 °C. The following mechanisms can explain the thermal decomposition of ozone:

$O_3 + M \leftrightarrow O + O_2 + M$

$O_3 + O \rightarrow 2O_2$

where M represents a third-body weighted sum of all the substances in the air, including O_3, O_2, N_2, CO_2, and He, and it is necessary to take away the energy of reaction (Benson, 1959; Zaslowsky et al., 1960). However, the gaseous ozone is characterized by different half-life times ($t_{1/2}$), depending on the temperature: 3 months at -50 °C, 18 days at -35 °C, 8 days at -25 °C, 3 days at 20 °C, 1.5 h at 120 °C, and 1.5 s at 250 °C (Batakliev et al., 2014).

Table 4 The decomposition process of ozone in water (Gottschall et al., 2010; Sehested et al., 1991; Staehelin & Hoigne, 1985).

Initiation	
Decomposition of ozone takes place by the presence of HO^- molecule; the $HO_2^{\bullet}$ radical establishes an equilibrium with the products of its split.	$O_3 + HO^- \rightarrow O2^{\bullet-} + HO_2^{\bullet}$ $HO_2^{\bullet} \rightarrow O_2^{\bullet-} + H^+$
Propagation	
$O2^{\bullet-}$ and $HO_2^{\bullet}$ radical ions are involved in a series of reactions; the formation of $HO_2^{\bullet}$ radical at the end can initiate propagation reactions again with the formation of superoxide radical ions ($O_2^{\bullet-}$).	$O_2^{\bullet-} + O_3 \rightarrow O_3^{\bullet-} + O_2$ $O_3^{\bullet-} + H^+ \leftrightarrow HO_3^{\bullet}$ $HO_3^{\bullet} \rightarrow HO^{\bullet} + O_2$ $HO^{\bullet} + O_3 \leftrightarrow HO_4^{\bullet}$ $HO_4^{\bullet} \rightarrow HO_2^{\bullet} + O_2$
Termination	
Radical catchers (radical scavengers) react with radicals forming secondary radicals without producing superoxide radical ions.	$HO^{\bullet} + HO_2^{\bullet} \rightarrow O_2 + H_2O$ $HO_4^{\bullet} + HO_4^{\bullet} \rightarrow H_2O_2 + 2O_3$ $HO_4^{\bullet} + HO_3^{\bullet} \rightarrow H_2O_2 + O_3 + O_2$

1.4 Air ionizers: tools and features

In the previous paragraphs, the principle for generating NAI has been described, highlighting the active species produced during the ionization process. Corona discharge has been identified as the common main principle for the generation of NAI, however different tools from different manufacturers were used by the researchers in their studies for generating NAI. Some models found in the literature are reported in Figure 3. In general, the devices used for the generation of ions are of small dimensions and therefore they can be easily placed in a treatments chamber of reduced volumes (Kampmann et al., 2009; Seo et al., 2001). Moreover, they are not expensive, the species generated are not dangerous for the operator, and they are easy to use.

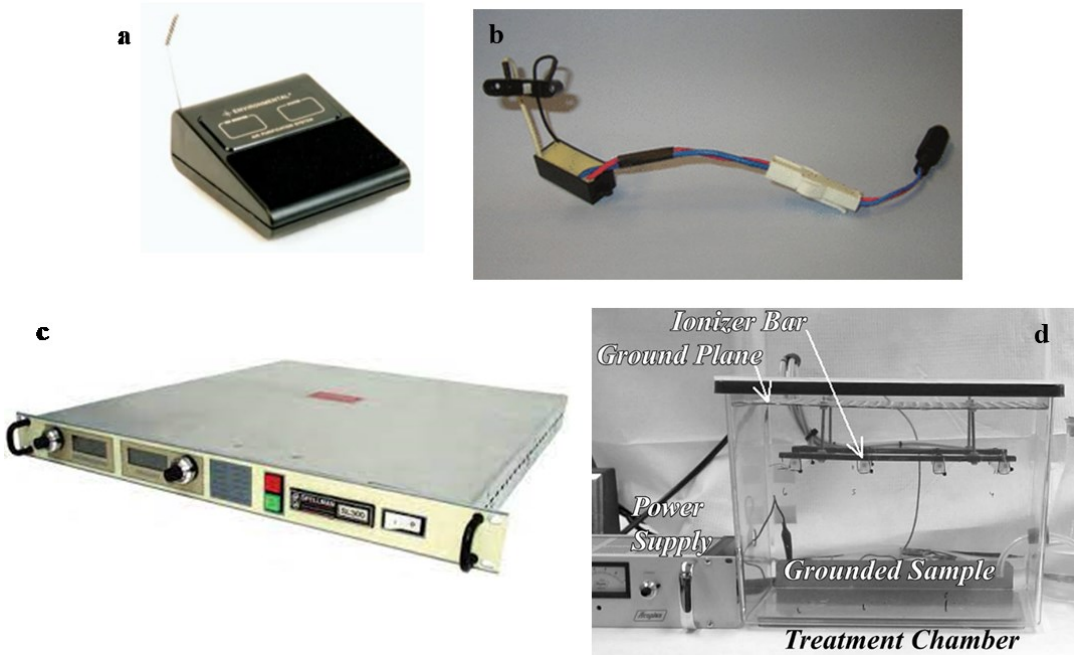

Figure 3 Examples of ion generators: Model VI-2000 Wein Products (a) Kong Hong ionizer (b), Spellman model SL300 (c), ESCS (d) (Arnold et al., 2004; L. Fan et al., 2007; Kampmann et al., 2009; Wu & Lee, 2004).

For example, L. Fan et al. (2002) have used Model VI-2000 negative ionizer (Wein Products, Inc., Los Angeles, CA), which has a spiral emitter that produces negative ions in intermittent pulses. Kong Hong ionizer (Xi'an Kong Hong Information Technology Co., Xi'an, Shaanxi, China) used by Kampmann et al. (2009) consists of a power supply that converts the main AC voltage into a smoothed DC voltage providing

the ionizer with the necessary input voltage of 12 V DC; the negative high voltage between the electrodes was -4.5 ± 0.5 kV DC. Wu & Lee (2004), to oxidize VOCs, have used a Spellman high voltage power supply (model SL300, USA): its circuitry includes a resonant high-frequency inverter which provides operation in transient and arcing environments. The operating ranges are between 0 and 50 °C for the temperature and the between 10 and 90% for relative humidity. Another ionizer used for the study of the antimicrobial activity was the ESCS (Electrostatic Space Charge System), which consists of an array of four -30 kV DC bars 13 cm long with seven sharp pointed electrodes each and an expanded metal ground plane 7.6 cm above the electrode points. Other examples of ionizer tools are Plasmater 2.5G (LG Electric Company, Seoul, Korea) with a power pack input voltage of 12 V DC and an output voltage peak to peak of ±2.8 kV (Park et al., 2016), WM 120 model (Air Ion Technologies Limited, New Milton, UK) that is a current unipolar air ionizer with an electrode potential of -5 kV (Shepherd et al., 2010), Electronic Air Purifier Escort that electrodes configuration as sharp emitter points (Tyagi et al., 2008).

1.5 Antimicrobial action of ionization technology

After reviewing everything related to ionization technology (the generation of NAI, the composition of NAI, the presence of ozone, and its properties) an overview of the mechanisms involved in the antimicrobial process should be done. NAI have been mostly studied and used for the improvement of the air quality in domestic and industrial environments (Jiang et al., 2018). Air ionization systems can be installed in house, office, institutional, commercial, and industrial locations for air cleaning since small ions and ion clusters can collide and react with any air impurity. Cluster ions electrically charge Particulate Matter of different sizes (PM_x) like environmental tobacco smoke, pollen, and dust facilitating their removal by filtration, and chemically react and destroy volatile organic compounds (VOCs), as odors (Daniels, 2002). Wu & Lee (2004) have investigated the reaction of NAI (concentration greater than 10^6 ions/cm^3) and three species of VOCs (chloroform, toluene, and 1.5-hexadiene) at 0, 25, and 70% RH. They have observed that, in all cases, the reaction kinetics were slow and complicated because of the smaller concentration of NAI than VOCs, suggesting that the ionization time must be a function of the number of particles. Moreover, relative

humidity influenced the NAI reaction with toluene, since the reaction rate constantly declined as the relative humidity increased. In addition to air cleaners and air conditioners, corona-discharge ionizers are used to treat biological contaminants, such as microorganisms (Y. S. Kim et al., 2011). At this time, there are three mechanisms known involved in the ionization that can cause microbial death (Fletcher et al., 2007):

1 electrodynamic effect due to ions, electrons, and other ionizing radiations;
2 electrostatic effect due to electric charge and electric field;
3 electrochemical effect due to ozone production.

Some authors state that NAI and ozone released during the ionization process have a synergistic effect on microbial cells (L. Fan et al., 2002; Kampmann et al., 2009; Tanimura, 1997). To distinguish between them, Fletcher et al. (2007) have investigated these phenomena on *Staphylococcus aureus*, *Mycobacterium parafortuitum*, *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, *Burkholderia cenocepacia*, *Bacillus subtilis*, and *Serratia marescens*. They have concluded that the main cause of cell death was exposure to ozone, with electroporation playing a secondary role. On the contrary, Y. S. Kim et al. (2011) found that the electrodynamic disruption had a primary role in the antibacterial effect: even if the concentration of ozone was below the detection limit of 0.01 ppm, the SEM images of *Escherichia coli* and *Staphylococcus epidermidis* showed broken cells and eroded or split cell surfaces, suggesting that this effect was due primarily to the presence of ions. Park et al. (2016) showed the bactericidal effect of an ionizer that generated a very low quantity of ozone (35 ppb detected in the treatment chamber) on *Escherichia coli*, *Enterococcus faecalis*, *Bacillus subtilis*, and *Staphylococcus aureus*. To make evident the bactericidal effect of ions alone, Noyce & Hughes (2002, 2003) have conducted two studies in which corona discharge took place in an oxygen-free environment to avoid the generation of ozone. They have demonstrated that both NAI and PAI were able to reduce the microbial load of *Escherichia coli* and *Pseudomonas veronii* at different operating conditions of voltage, current, and time. In light of this reviewed literature, a conclusive statement on the mechanism involved primarily in the bactericidal effect can not be done. Anyway, the effect of ions and ozone on microbial cells are similar. Regarding the ionization process, electrostatic interactions between air ions and charged groups in the cell wall could induce a physiological change in the cell wall structure. Ions generated during a

treatment chemically react with the microbial cell through free-radical mechanisms leading to the destruction of the cell and, therefore, to its death (Digel et al., 2005). Park et al. (2016) have found that oxidative stress induced by exposure to negative and positive ions could result in the production of intracellular reactive oxygen species (ROS), which targets are deoxyguanosine, a DNA component, and aconitase, an enzyme involved in the tricarboxylic acid cycle. Therefore, also ions as ozone induce oxidative stress and damage, causing cell death. Several studies have been conducted to evaluate the antimicrobial properties of NAI treatment as a function of different environmental conditions. Seo et al. (2001) have tested the effect of high levels (unknown) of NAI generated by a custom electrostatic space charge system on an aerosol containing *Salmonella enteritidis* inside a closed box. After 3 h period of treatment, a reduction of 2 Log was observed both on the agar surface and aerosol. Fan et al. (2002) have tested the viability of *Pseudomonas fluorescens*, *Erwinia carotovora* pv. *carotovora*, and *Escherichia coli*, grown on both Potato Dextrose Agar (PDA: potato infusion, glucose) and Nutrient Agar (NA: beef extract, peptone), after the treatment at different conditions (ozone alone, NAI alone, and ozone and NAI together). The concentration of ozone was 0.1 ppm, while that one of NAI was 10^6 ions/cm^3. Results indicated that the cell death of the three microorganisms was more rapid on PDA than NA, indicating that the bactericidal action of NAI was dependent on the medium in the same way as ozone. The authors have not obtained an effect of NAI alone, but they found that the bactericidal effect of ozone and NAI together was greater than ozone alone demonstrating a synergism between the two treatments. Arnold et al. (2004) have tested the use of ESCS (Electrostatic Space Charge System) that generated NAI at a concentration of 10^6 ions/cm^3 minimizing the production of ozone at levels below 0.01 ppm on five pathogens biofilms formed on a stainless steel surface and bacterial spores of *Bacillus stearothermphilus*. The microorganisms involved in the study were *Campylobacter jejuni*, *Escherichia coli*, *Salmonella enteritidis*, *Listeria monocytogenes*, and *Staphylococcus aureus*; after 3 h of ionization, the Log reduction was 3.45, 1.48, 2.75, 3.70, and 1.98 respectively. Regarding the effect on spores, NAI induced a Log reduction of 2.43 and 0.55 after 3 and 6 h treatment, respectively. Tyagi et al. (2008) have investigated the effect of NAI at a concentration greater than 10^6 ions/cm^3 on *Pseudomonas fluorescens* and *Escherichia coli*, finding that the action of NAI on

microbial cells depends on the cell surface characteristics and physiological conditions induced by the surrounding environment. For example, they have observed that the starved cells were become more resistant to NAI compared to the cells grown in normal conditions, or that the viability reduction was higher for the Tryptone Soya Agar medium (TSA: casein enzymatic hydrolysate, an enzymatic digest of soya bean, and dextrose) than NA medium and King's medium (glycerol).

Table 5 The use of NAI for the treatment of food.

Food	Treatment conditions	Description	References
Mandarin oranges	1 – 5 ppm ozone 10^4 – 10^5 NAI/cm^3	Fruit decay in the treated sample was 2% compared with 25% of the untreated fruit.	(Li et al., 1989)
Potato slab	3.02x10^{12} PAI/cm^2 s 7.31x10^{12} NAI/cm^2 s	Increase of evaporation rate of 3 times than air-drying without ions.	(Y. H. Chen & Barthakur, 1991)
Grapes	50 ppb ozone 5x10^4 NAI/cm^3	Little growth of molds during 8 months of storage at optimum temperature.	(Tanimura et al., 1998)
Onions	0 – 250 ppb 1x10^6 – 9x10^6 NAI/cm^3	Reduction of airborne mold spores; modestly discoloration of surface; no changes in internal decay, firmness, sprouting, or rooting.	(J. Song et al., 2000)
Grapes **Carrots**	0.1 – 0.5 ppm ozone 2x10^4 – 7x10^4 NAI/cm^3	Grapes: decay reduction by 40% during 3 weeks of storage at 10 °C. Carrots: growth rates reduction of *Botrytis cinerea* (69%) and *Sclerotinia sclerotiorum* (57%).	(Forney et al., 2001)
Mung bean seeds **Apple fruit**	ESCS system for NAI generation (data not shown)	Mung bean seed: no reduction of *Escherichia coli* after 18 h of treatment. Apples: 0.9 Log reduction of *Escherichia coli* on whole apples, but no reduction in sliced apples after 3 h of treatment.	(X. Fan et al., 2007)
Lettuce	1.0x10^6 ions/cm^3 (high) 1.5x10^5 ions/cm^3 (medium) 8.0x10^3 ions/cm^3 (low)	No changes in ascorbic acid, saccharide, and organic acid contents at three dosages; lower water loss rate and higher chlorophyll content with NAI treatment at low dosage.	(Tamaki & Uyama, 2008)
Strawberries **Tomatoes**	0.5 ppm ozone 6.1x10^6 NAI/cm^3	Reduction of epiphytic flora (bacteria and yeast) after15 days of exposure to NAI and ozone; suppression of visible mycelia and spore production (*Botrytis cinerea* and *Penicillium expansum*).	(Tuffi et al., 2012)
Red leaf lettuce	70x10^4 ions/cm^3 (high) 19x10^4 ions/cm^3 (medium) 10x10^4 ions/cm^3 (low)	Application in plant factories. Vigorous growth after 4 weeks of treatment; improvement growth characteristics (leaf area, shoots fresh weight) with medium and high levels of NAI.	(M. Song et al., 2014)
Cauliflower	428 mg/m^3 of ozone NAI (data not shown)	Reduction of *Listeria innocua* (2.0 Log), *Escherichia coli* (2.8 Log), and mesophilic bacteria (1.4 Log) after 7 days of treatment at 4 °C.	(Boumail et al., 2016)

1.6 Ionization technology for the preservation of food at refrigeration conditions

Although the literature has available a good number of papers related to the study of the antimicrobial effect of ionization technology *in vitro*, the research remains still poor about the evaluation of this technology for a possible application for food preservation. Table 5 displays the literature in which the use of NAI has been applied for the treatment of food. It is possible to notice that the experimentations on food with the use of ionization technology are quite recent and concern the study of fruits and vegetables. In most cases, only negative and not positive air ions have been considered for the treatment, and ozone, even if at low concentrations, was almost always present. Generally, the treatment with NAI has been able to improve the decay of fruits and vegetables, like grapes or carrots, reducing the decay by 40% during 3 weeks of storage at 10 °C (Hildebrand et al., 2001). In the case of lettuce, the presence of NAI resulted to be favorable to the maintenance or improvement of the quality characteristics, like the contents of ascorbic acid and chlorophylls, and to stimulate the growth characteristics as the leaf area during the production in the plant factories (M. Song et al., 2014; Tamaki & Uyama, 2008). Moreover, the use of NAI was resulted in an efficient treatment to control the growth rate of bacteria, yeasts, and molds also during the storage at refrigeration temperature (Boumail et al., 2016; Tanimura et al., 1998; Tuffi et al., 2012). The possibility to use ionizer modules at refrigeration temperature can be considered an important outcome, considering that the use of refrigerated storage of fresh foods like fruits or vegetables after harvests is one of the most common methods used for their preservation, especially in the domestic environment. Indeed, the natural microflora present in the food causes the decay of the products but it can also be the source of cross-contamination between food and surfaces during the preservation period (Ye et al., 2019). To control these events, the use of NAI can be used for the improvement of the hygienic status of the refrigerators (Kampmann et al., 2008, 2009). However, additional factors must be considered for the correct use of the ionizers inside a refrigerator, like a temperature and relative humidity. For example, the technical specification of the Spellman high voltage power supply (model SL300, USA) (Wu & Lee, 2004) displays the usage ranges of temperature (between 0 and 50 °C) and relative

humidity (between 10 and 90%) to underline the importance to observe these parameters for guarantying the correct functioning and, therefore, to obtain the expected performances of the device.

Most of the information present in the introduction can be found in:
Baggio, A., Marino, M., Innocente, N., Celotto, M., & Maifreni, M. (2020). Antimicrobial effect of oxidative technologies in food processing: an overview. *European Food Research and Technology*, 246(4), 669-692. https://doi.org/10.1007/s00217-020-03447-6.

AIM AND OUTLINE OF THE PH.D. THESIS

The present Ph.D. thesis aimed to understand if ionization technology can be used inside specific areas of a domestic refrigerator to improve the hygienic status of the surrounding environment without causing adverse effects on food. To reach the final purpose, different steps have been followed to investigate the effect of technology from the most controlled model systems to the more variable systems that represent real situations.

Thus, the work of the Ph.D. project can be divided into three parts. The outline is reported in Table 6.

Part 1. Evaluation of the antimicrobial effect of ionization technology on model systems.

Three ionizers different for the combination of NAI and ozone produced were used based on the characteristics reported in the relative datasheets. Firstly, the devices have been characterized to verify the statements concerning the amount of NAI generated and the ozone contextually released. Thus, the antimicrobial effect was assessed in a controlled environment at room and refrigeration temperatures using six food-related microorganisms.

Part 2. Evaluation of the ionization technology effect on food.

The assessment of the ionization effect on food was done with the selected devices, in the same controlled environment realized for the part on model systems, using some food categories. The evaluation was carried out through the color changes and the modification of natural microflora during the period of the exposition to the treatment at refrigeration temperature.

This part has allowed selecting the ionizer with the lowest amount of ozone production as the device that cannot negatively affect the quality of the tested food in terms of visual appearance and color changes.

Part 3. Study of the selected ionizer in a simulated real application inside a domestic refrigerator.

The previously selected ionizer has been tested firstly to evaluate the antimicrobial potential on three different surfaces that typically represent the materials of a domestic refrigerator. Thus, the ionizer has been used to study more in deep the effect of ionization on three selected food types (lettuce, strawberry, and mushroom) through the analysis of the color change, the natural microflora, and other chemical-physical parameters.

Table 6 Outline of the Ph.D. thesis.

		Ionizer	Activity	Examination	Outcomes/Decisions
Part 1	Step 1	DA DB	Study of the antimicrobial effect on model systems at room temperature	Comparison of the antimicrobial effect through the selection of specific microorganisms	DB was rejected because of the poor intrinsic characteristics → Analyze DA on additional microorganisms at room and refrigeration temperatures
	Step 2	DA	Study of the antimicrobial effect on model systems at room and refrigeration temperatures	Comparison of the antimicrobial effect at the two temperatures	Antimicrobial effect for all species at both temperatures
	Step 3	DM	Study of the antimicrobial effect on model systems at room and refrigeration temperatures	Comparison with DA of the antimicrobial effect through the selection of specific microorganisms	Increase the period of the exposure with DM to investigate the antimicrobial effect for prolonged times at refrigeration temperature
	Step 4	DM	Study of the antimicrobial effect on model systems at refrigeration temperatures increasing the exposure times	Evaluation of antimicrobial effect at longer exposure times	Increase the period of the exposure with DM at refrigeration temperature can improve its antimicrobial effect
Part 2		DA DM	Preliminary assessment of ionization effect on food	Comparison of the ionization effect on food through color and microbiological analysis	Use DM as the best device for the preservation of food visual qualities at refrigeration temperature
Part 3	Step 1	DM	Assessment of antimicrobial effect on surfaces at refrigeration temperature	Evaluation of ionization effect in a simulation of real application	DM could maintain or improve the hygienic status of the fridge surfaces without negatively affect food
	Step 2		Assessment of ionization effect on selected food at refrigeration temperature		

PART 1

ANTIMICROBIAL EFFECT OF IONIZATION TECHNOLOGY ON MODEL SYSTEMS

Negative air ionization was commonly used for the improvement of the air quality. However, also the effect towards microorganisms was recently observed and therefore it could represent a promising non-chemical and non-thermal technology for reducing microbial population improving the quality of air and surfaces of specific environments. The small dimensions of the devices and the ease and safety of use make ionization a possible technology for the application in confined volumes or small appliances intended for the storage of the food, like a refrigerator.

2 USE OF DIFFERENT AMOUNTS OF NAI AND OZONE TO ACHIEVE ANTIMICROBIAL EFFECTS ON FOOD-RELATED MICROORGANISMS

2.1 Introduction and aim of the study

In recent years, air ionization systems have been proposed for the cleaning treatment of air and surfaces. It is considered a rapid, green, and low-cost strategy as ions react and destroy air pollutants, such as volatile organic compounds, dust, tobacco smoke, and other odors, maintaining a better air quality (K. H. Kim et al., 2017; Y. S. Kim et al., 2011). In addition to air cleaners and conditioners, this technology is demonstrated to be effective also in the treatment of biological contaminants, microorganisms as well. The antimicrobial effect of NAI has been evaluated towards different microorganisms related to the food industry, like *Salmonella enteritidis*, *Pseudomonas fluorescens*, *Erwinia carotovora* pv. *carotovora*, *Escherichia coli*, bacterial spores of *Bacillus stearothermophilus*, *Campylobacter jejuni*, *Listeria monocytogenes*, and *Staphylococcus aureus* (Arnold et al., 2004; L. Fan et al., 2002; Seo et al., 2001; Tyagi et al., 2008). These studies have demonstrated that the effect of NAI in the reduction of viability was dependent on the microbial strain, the environmental conditions, and the characteristics of the device used for the generation of NAI. Moreover, the concentration of produced ions depends on the surrounding operating conditions like relative humidity, temperature, and distance from the source (Wu, Lee, Yang, Yu, & Lou 2006). Nowadays, some new devices are developing to further improve NAI generation reducing at the same time the release of ozone (Jiang

et al., 2018) since it can be toxic at certain levels (Pascual et al., 2007), showing that the synergism between NAI and low levels of ozone have an antimicrobial effect (Arnold et al., 2004; Hildebrand et al., 2001; Kampmann et al., 2009; Seo et al., 2001; Tuffi et al., 2012).

The aim of the study was to investigate the antimicrobial effect of ionization technology towards *B. subtilis*, *E. coli*, *L. monocytogenes*, *Ps. fluorescens*, *P. roqueforti*, and *S. cerevisiae* on model systems through the exposition to different concentrations of NAI and ozone.

2.2 Materials and methods

2.2.1 Microbial cultures

Six microorganisms were used in the present study: *Bacillus subtilis* DSMZ 4181, *Escherichia coli* ATCC 8048, *Listeria monocytogenes* 284 and *Pseudomonas fluorescens* L22 (wild type strains belonging to the collection of DI4A, University of Udine), *Penicillium roqueforti* PR N (used for the preparation of blue cheese), and *Saccharomyces cerevisiae* SCHLB (used for the preparation of fermented milk). Stock cultures of the strains were stored at -80 °C in the relative medium added of glycerol at 30% (v:v). Before the experiments, microbial suspensions were prepared transferring a portion of stock cultures in the relative broth. The suspensions were incubated overnight twice at 37 °C for *E. coli* and *L. monocytogenes*, at 30 °C for *B. subtilis, Ps. fluorescens,* and *S. cerevisiae,* and at 22 °C for *P. roqueforti* reaching a final concentration of the overnight of 10^8-10^9 CFU/mL.

2.2.2 Culture media

Brain Heart Infusion (BHI, Oxoid, Milan, Italy) was used to prepare the suspensions of *B. subtilis*, *E. coli*, *L. monocytogenes*, and *Ps. fluorescens* and the agar plates for the spread of *L. monocytogenes*. Plate Count Agar (PCA, Oxoid, Milan, Italy) was used to prepare the solid medium for the spread of *B. subtilis*, *E. coli*, and *Ps. fluorescens*. Malt Extract (ME, Oxoid, Milan, Italy) broth and agar were used to cultivate *P. roqueforti*. Yeast extract Peptone Dextrose (YPD, Sigma Aldrich, Milan, Itlay) broth and agar were used for *S. cerevisiae*.

2.2.3 Preparation of the samples

From the overnight suspensions (10^8-10^9 CFU/mL), seven serial decimal dilutions of each strain were done in Maximum Recovery Diluent (MRD, Oxoid, Milan, Italy) and plated on the relative agar medium according to the drop plate method (Herigstad et al., 2001). Therefore, each sample consisted of two agar plates containing the overnight suspension and all its seven dilutions.

2.2.4 Operative conditions

Three negative air ion generators available at the company were from Aquapure, Beko, and Murata manufacturers. Conventionally, the Aquapure ionizer was named device A (DA), Beko ionizer was named device B (DB), and Murata ionizer was named device M (DM), (this nomenclature is adopted for all the chapters of the thesis). They were characterized to verify the statements reported in the technical data sheet (Table 7) concerning particularly the amount of NAI generated and the simultaneous amount of ozone released.

Table 7 The main characteristic of DA, DB, and DM reported in the technical datasheets.

	DA	**DB**	**DM**
Description	Electronic housing linked with the load part – made by grid and needles-through high voltage silicon cables	Plastic housing containing the ground and the needle electrodes	Compact generator made by a ground electrode and a discharge needle electrode
Generation principle of NAI and ozone	Corona discharge	Corona discharge	Corona discharge
Input voltage	230 V AC	230 V AC	12 V DC
Output voltage	-3.7 – -4.7 kV DC	-4.2 – -5.2 kV DC	-2.5 – -3.5 kV DC
Generation of NAI (at 30 cm from the source)	≥ 6.0 x 10^6 NAI/cm^3	10^6 NAI/cm^3	7.2 x 10^6 NAI/cm^3
Generation of ozone	0.10 mg/m^3*	0.30 mg/h	0.04 mg/h
Usage temperature	-10 – +60 °C	NA**	NA**
Usage relative humidity	< 90% RH	NA	NA

*expressed as active oxygen concentration (volume of measurement not reported in the datasheet)
**NA = data not available in the technical datasheet

The number of NAI was measured by the Air Ion Counter Model AIC2 (AlphaLab Inc., Salt Lake City, UT, USA), which drew in the air at the top and let it out on the bottom calculating the number of ions twice per second. The amount of ozone was estimated by

the Ozone Analyzer Model UV-100 Serial (Eco Sensors, Santa Fe, NM, USA), which used a mercury lamp filtered for absorption at 254 nm. The evaluation of the production of NAI and ozone was done inside the air-tight chamber following used for the treatments (Figure 4) with and without the presence of inoculated plates.

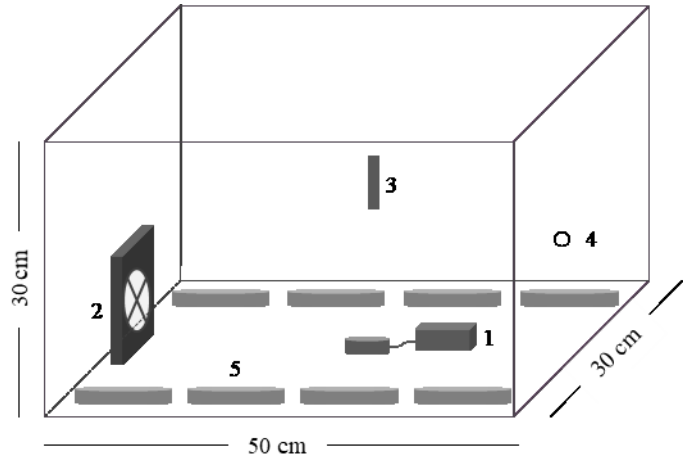

Figure 4 Setup realized for the characterization of the NAI generators. 1 = device, 2 = fan, 3 = T-RH sensor, 4 = hole for monitoring ozone, 5 = inoculated plates. The same setup was used for the ionization treatments.

2.2.5 Preparation of the treatment chamber

For the treatments, an air-tight chamber made up of plastic material was used (size 30 cm x 50 cm x 30 cm; H x L x W). The total volume of the chamber was 0.045 m^3. Before the exposure to the ionization treatment, the chamber was cleaned with ethanol 70% (v:v). Inside the chamber, the device was settled on the base in the center. A fan (NMB 3610RL-04W-B10, Minebea Co., Ltd), having a maximum airflow of 1.13 – 1.84 m^3/min and a rating voltage of 12 V DC, was placed on the base near the device to guarantee the air circulation. On one side of the chamber, a sensor (AMS CCS811) was fixed to monitor continuously the values of temperature and relative humidity during the treatments.

2.2.6 Ionization treatment on model systems

DA and DB were available at the company. Successively, also DM was selected considering the intrinsic characteristics of the device, especially the amounts of NAI and ozone generated. Based on the availability of the devices, the experimental design of the model systems tests resulted at the end divided into four experiments (Figure 5).

Experiment 1. The antimicrobial effect of DA and DB was studied on *L. monocytogenes* and *Ps fluorescens* for 1, 2, 3, 4, 5, 6 h at room temperature (roomT).

Experiment 2. The antimicrobial effect of DA was investigated on *B. subtilis*, *E. coli*, *L. monocytogenes*, *Ps. fluorescens*, *P. roqueforti*, and *S. cerevisiae* for 1, 2, 3, 4, 5, and 6 h at roomT and refrigeration temperature (refT).

Experiment 3. The antimicrobial effect of DM was assessed on *L. monocytogenes* and *Ps. fluorescens* for 1, 2, 3, 4, 5, and 6 h at roomT and refT.

Experiment 4. The antimicrobial effect of DM was studied for *B. subtilis*, *E. coli*, *L. monocytogenes*, *Ps. fluorescens*, *P. roqueforti*, and *S. cerevisiae* for 4, 16, 24, and 72 h at refT.

The samples prepared as reported in section 2.2.3 were placed on the base of the chamber and removed from their lid (Figure 4). The desiccating effect was determined by placing the samples, inside the air-tight chamber equipped only with the fan. The conditions of exposure (time and temperature) were the same as the treatment for each experiment. The purpose was to assess the influence of flowing air on the vitality of the microorganisms; these samples represented the negative control samples (Park et al., 2016). When the tests were carried out at refT, additional controls at roomT were kept assessing the effect of the exposure to cold temperature. After the treatment, the plates were removed from the chamber, closed with the lid, and incubated at specific conditions (*E. coli* and *L. monocytogenes* at 37 °C for 24 h, *B. subtilis*, *Ps. fluorescens* and *S. cerevisiae* at 30 °C for 24 h, and *P. roqueforti* at 22 °C for 72 h). Finally, the count of viable cells was done.

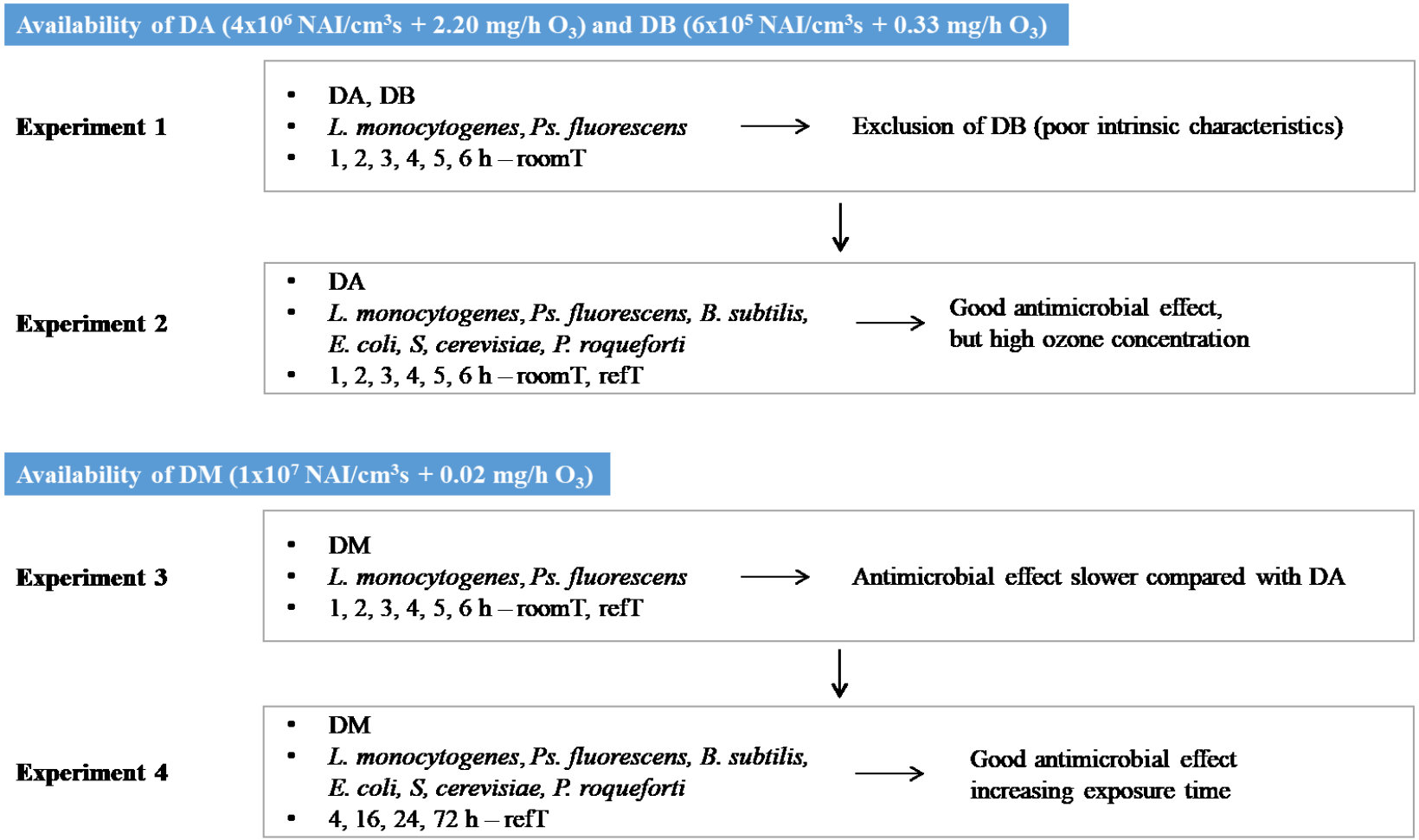

Figure 5 Experimental design of the model systems tests.

2.2.7 Statistical analysis

Each trial was carried out in two biological replicates and for each of these, two technical repetitions were done. The results were expressed as Log (N/N_0), where N = viable count after the treatment and N_0 = viable count of the negative controls. The means obtained from replicate tests were subjected to one-way analysis of variance ($p < 0.05$), preceded by the Levene test to verify the homogeneity of variance between means using Statistics 8.0 (Statsoft software, Tulsa, Oklahoma, USA). Differences between the means were assessed using Tukey's HSD post-hoc test.

2.3 Results and discussion

2.3.1 Characterization of devices

Table 7 reported the main characteristics of DA, DB, and DM used for the study. The characterization was carried out inside the 0.045 m^3 air-tight chamber. The volume selected represented an intermediate value than those usually reported in other studies conducted using NAI technology (Arnold et al., 2004; Chauhan et al., 2015; X. Fan et al., 2007; Kampmann et al., 2009; Park et al., 2016; Tyagi et al., 2008; Tyagi & Malik, 2010, 2012). The characterization was done considering two different setups. The first setup (setup 1) was realized by placing inside the air-tight chamber only the device and the fan, to estimate the amount of NAI and ozone generated excluding adverse conditions, such as the presence of matters that could interact with the species generated. This allowed to verify the effective potentiality of the devices. The second setup (setup 2) was arranged by adding the inoculated agar plates, to simulate the conditions in which the tests would then be conducted. This setup aimed to study the trend of NAI and ozone in presence of the microorganisms and organic and inorganic substances contained in the agar medium composition since it is known their interaction with ozone (Güzel-Seydim et al., 2004; Marino et al., 2015; Restaino et al., 1995) and NAI (Fletcher et al., 2007; Noyce & Hughes, 2002, 2003). Moreover, the characterization of the devices was done at roomT (DA, DB, and DM) and refT (DA and DM) to evaluate how this parameter influenced the devices during the working. The values of temperature measured in the air-tight chamber were 22.8 ± 1.1 °C at roomT and 5.4 ± 0.7 °C at refT. The relative humidity detected inside the chamber was 75 ± 3% RH in setup 1 and 89 ± 1% RH in setup 2, probably

higher due to the presence of agar medium. The relative humidity value seemed to be strictly correlated with the amount of NAI present in the chamber; it was observed that an increase in the air humidity led to a significant increase in the number of negative ions in the air, due to the formation of cluster ions with water molecules (Schiessling et al., 2018). The values detected resulted to be within the ranges reported in the datasheet of DA (Table 7); the same ranges were thus considered valid also for DB and DM, for which no specifications were reported about this parameter.

For the characterization of the devices, the interest was primarily focused on the values of NAI and ozone generated, being the active species considered responsible to give the antimicrobial effect. However, a significant electrical outcome emerged for DB: it resulted to have a relevant change in performance over alternating current input voltage variation. Despite in the datasheet the input voltage was 230 V AC, it stopped completely to work at about 160 V AC input showing relevant variation (> 10%).

NAI. DA, DB, and DM resulted to produce ions in the range of $1x10^6$ – $7x10^6$ NAI/cm^3s (mean $4x10^6$ NAI/cm^3s), $2x10^5$ – $1x10^6$ NAI/cm^3s (mean $6x10^5$ NAI/cm^3s), and $8x10^6$ – $2x10^7$ NAI/cm^3s (mean $1x10^7$ NAI/cm^3s) respectively, confirming the values of the technical datasheets. Generally, it was observed that the amount of NAI resulted remain almost constant during the overall period of 6 hours for each setup and temperature considered in the characterization. This was due to the short lifetime of NAI that is 100-1000 s in clean air, which allowed maintaining their average concentration unchanged over time (Gaunt et al., 2005). This behavior was very important since it was demonstrated that a constant production of more than 10^6 ions/cm^3 is needed to permit an antimicrobial effect (L. Fan et al., 2002; Tyagi et al., 2008).

Table 8 Values of ozone (mg/m^3) detected with setup 1 and setup 2 inside the air-tight chamber during the characterization of DA, DB, and DM at roomT and refT (mean ± SD).

		Setup 1		Setup 2	
	h	**room T**	**ref T**	**room T**	**ref T**
DA	**1**	$52.2 \pm 2.70^{aA*}$	48.8 ± 2.40^{aA}	1.50 ± 0.36^{aB}	3.64 ± 0.06^{aA}
	2	92.2 ± 4.30^{bA}	83.0 ± 1.80^{bB}	1.38 ± 0.14^{aB}	4.90 ± 0.12^{bA}
	3	123 ± 3.70^{cA}	107 ± 1.10^{cB}	2.08 ± 0.24^{aB}	5.02 ± 0.08^{bA}
	4	147 ± 3.70^{dA}	134 ± 3.50^{dB}	2.24 ± 0.50^{aB}	5.54 ± 0.08^{bcA}
	5	167 ± 1.40^{eA}	150 ± 2.60^{eB}	2.62 ± 0.80^{aB}	6.08 ± 0.24^{cdA}
	6	179 ± 1.90^{fA}	161 ± 0.40^{fB}	2.74 ± 1.06^{aB}	6.88 ± 0.30^{dA}
DB	**1**	7.23 ± 3.51^{a}	-**	3.28 ± 0.12^{a}	-
	2	13.97 ± 5.49^{ab}	-	3.43 ± 0.03^{a}	-
	3	19.21 ± 6.59^{bc}	-	3.54 ± 0.10^{a}	-
	4	24.59 ± 7.61^{cd}	-	3.43 ± 0.09^{a}	-
	5	28.8 ± 8.00^{de}	-	3.60 ± 0.08^{a}	-
	6	32.8 ± 8.20^{e}	-	3.69 ± 0.03^{a}	-
DM	**1**	0.40 ± 0.10^{aA}	0.38 ± 0.22^{aA}	***ND	ND
	2	1.34 ± 0.08^{bA}	1.44 ± 0.08^{bA}	ND	ND
	3	1.86 ± 0.06^{cA}	1.90 ± 0.08^{cA}	ND	ND
	4	3.00 ± 0.10^{dA}	2.76 ± 0.08^{dB}	ND	ND
	5	3.32 ± 0.10^{eB}	3.56 ± 0.08^{eA}	ND	ND
	6	4.54 ± 0.20^{fA}	4.44 ± 0.16^{fA}	ND	ND

*Means with different lowercase letters within a column are significantly different ($p < 0.05$) for the same setup; means with different uppercase letters within a row are significantly different ($p < 0.05$).
**- Investigation not performed.
***ND: not detected (<0.02 mg/m^3, the detection limit of the ozone analyzer).

Ozone. The results of the ozone characterization of the devices are reported in Table 8. By the study of setup 1, which the aim was to know the amount of ozone generated, DA resulted in producing 2.20 mg/h of ozone, DB 0.33 mg/h, and DM 0.02 mg/h with an accumulation of the gas inside the chamber during the 6 h of working for all the devices. The expression in mg/h is calculated from the concentration of the ozone after 1 h in the 0.045 m^3 chamber; this unit is correct when the aim is to express the production capacity and not a concentration value. For DB and DM the assessment confirmed the values reported in the technical datasheet. For DA emerged that the generation of ozone was higher than expected. In the datasheet, the active oxygen concentration (referring to the totality of the oxygen active species, i.e., singlet oxygen, oxygen atoms, and ozone) was reported, therefore the expected value of ozone should not have been greater than 0.0045 mg/h

considering the volume of the chamber used. For setup 2, considering the production rate (mg/h) of the devices stated with setup 1, the concentration (mg/m^3) of ozone resulted to be lower compared to the value that could have been measured if only the fan and the device were present in the chamber. This expected outcome confirmed what was already reported by Khadre et al. (2001) that is the presence of microorganisms and organic and inorganic matter interact with the ozone reducing its amount in the air. In the case of DA, the values increased over time, especially at refT (6.88 ± 0.30 mg/m^3 compared to 2.74 ± 1.06 mg/m^3 at roomT). This could be explained because the solubility of ozone in water (in this case, molecules of water present in the air) increases in decreasing the temperature (Khadre et al., 2001). The concentration of ozone detected during the setup 2 test with DB at roomT remained constant during the 6 h. In the case of DM, the concentration of ozone during the study with setup 2 was so low that it was not detected by the instrument (the detection limit of the ozone analyzer was 0.02 mg/m^3) in any hour of analysis, finding results comparable with the devices used by Arnold et al. (2004) and Seo et al. (2001). Concluding, three different conditions of production of NAI and ozone were identified:

1 DA = 4x10^6 NAI/cm^{3}s + 2.20 mg/h O_3;

2 DB = 6x10^5 NAI/cm^{3}s + 0.33 mg/h O_3;

3 DM =1x10^7 NAI/cm^{3}s + 0.02 mg/h O_3.

2.3.2 Antimicrobial effect of DA and DB at room temperature (experiment 1)

This part of the study was considered an explorative assessment to understand the potential antimicrobial effect given by the conditions identified for DA (4x10^6 NAI/cm^{3}s + 2.20 mg/h O_3) and DB (6x10^5 NAI/cm^{3}s + 0.33 mg/h O_3) on the microorganisms *L. monocytogenes* and *Ps. fluorescens*. These two bacteria were chosen from among all those used for the present experimentation since they are considered peculiar species of interest in the food industry. *L. monocytogenes* is a Gram-positive pathogen, cause of listeriosis in humans, ubiquitous as able to grow and survive in different environments, and psychrotrophic, having the capacity to grow at refrigeration temperature (-0.5 – 9.3 °C) (Ferreira et al., 2014; Walker et al., 1990). The interest of the food industry towards this microorganism resides also in the European mandatory limits for food (Reg. CE 2073/2005). *Ps. fluorescens* is Gram-negative, psychrotrophic, ubiquitous, typical

spoilage bacteria involved in many food-quality decays (fresh vegetables, meat and poultry, eggs, fish, and dairy products). Moreover, *L. monocytogenes* and *Ps. fluorescens* are known to be able to form biofilms in food processing environments (Marino et al., 2018) representing an important challenge for the hygienic conditions of foods and surfaces and consumer health (Møretrø & Langsrud, 2017).

The exposition to ionization treatment was done for 1, 2, 3, 4, 5, and 6 hours at roomT (22.8 ± 1.1 °C – 97 ± 3% RH) (Table 9). For all the times considered, a desiccating effect was not observed, and the vital count of the control samples remained always 10^8-10^9 CFU/mL.

Table 9 Effect of the treatment with DA ($4x10^6$ NAI/cm^3s + 2.20 mg/h O_3), DB ($6x10^5$ NAI/cm^3s + 0.33 mg/h O_3) on *L. monocytogenes* and *Ps. fluorescens* at roomT (22.8 ± 1.1 °C – 97 ± 3% RH). Data are expressed as Log reduction (mean ± SD).

	L. monocytogenes		***Ps. fluorescens***	
Exposure time (h)	**DA**	**DB**	**DA**	**DB**
1	1.85 ± 0.32 $^{a\,A*}$	2.04 ± 0.93 $^{a\,A}$	1.13 ± 0.87 $^{a\,A}$	0.14 ± 0.32 $^{a\,A}$
2	2.54 ± 0.28 $^{bc\,A}$	1.39 ± 0.75 $^{a\,A}$	1.84 ± 0.47 $^{ab\,A}$	0.10 ± 0.26 $^{a\,A}$
3	2.86 ± 0.04 $^{cd\,A}$	1.10 ± 0.04 $^{a\,B}$	2.87 ± 0.64 $^{ab\,A}$	0.48 ± 0.52 $^{a\,A}$
4	2.88 ± 0.17 $^{cd\,A}$	1.32 ± 0.62 $^{a\,B}$	3.21 ± 0.36 $^{ab\,A}$	0.11 ± 0.44 $^{a\,B}$
5	2.85 ± 0.24 $^{b\,A}$	2.25 ± 1.18 $^{a\,AB}$	3.72 ± 0.17 $^{b\,A}$	0.83 ± 0.58 $^{a\,B}$
6	3.03 ± 0.14 $^{d\,A}$	2.38 ± 1.04 $^{a\,B}$	4.04 ± 0.56 $^{b\,A}$	0.95 ± 0.67 $^{a\,B}$

*For each microorganism means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$); means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Generally, for both the devices and the microorganisms, the antimicrobial effect was observed to increase extending the period of the exposure to the treatment. However, the results have shown that DA was able to generate the highest antimicrobial effect for both microorganisms achieving after 6 h 3.03 ± 0.14 Log reduction for *L. monocytogenes* and 4.04 ± 0.56 Log reductions for *Ps. fluorescens*. In the same exposure time DB generated 2.38 ± 1.04 Log reductions for *L. monocytogenes* and 0.95 ± 0.35 Log reductions for *Ps. fluor*escens. Even if the results showed that DA and DB were both able to achieve an antimicrobial effect, only in the case of DA, which generates a higher amount of ozone (2.20 mg/h than 0.33 mg/h of DB), the reductions were significantly different ($p < 0.05$) increasing the period of exposure, bringing out what was previously found by other studies (L. Fan et al., 2002; Fletcher et al., 2007). In addition to the different concentrations

of ozone, during the characterization DB was resulted to have an electrical instability, that probably reflected in the quite low generation of NAI ($6x10^5$ NAI/cm^3s) and the high variability of the ozone generation. Effectively, the standard deviations obtained during the characterization of the devices with setup 1 (Table 8) were wide. Therefore, this instability was probably reflected in the antimicrobial performances of the device. Because of these considerations, DB was not considered for the following steps of the study.

2.3.3 Antimicrobial effect of DA at room and refrigeration temperature (experiment 2)

Analyzing the results obtained with experiment 1 about the antimicrobial effect of the exposition to different concentrations of NAI and ozone, the decision to exclude DB for the following steps of the study was taken. The attention was focused on DA which had stable electric performances that allow it to generate continuously $4x10^6$ NAI/cm^3s and 2.20 mg/h O_3. The interest was to extend the study to other food-related microorganisms having different own qualities. Four additional microorganisms were selected: *Bacillus subtilis*, *Escherichia coli*, *Saccharomyces cerevisiae*, and *Penicillium roqueforti*. *B. subtilis* is a spore-forming Gram-positive frequently reported as an important spoiler of heat-treated foods, dairy and bakery products, and fresh vegetables (Møretrø & Langsrud, 2017). *E. coli* is a Gram-negative pathogen commonly considered as an indicator of faecal contamination and cause of foodborne illnesses related to the consumption of fresh fruits and vegetables (X. Fan et al., 2007). *S. cerevisiae* and *P. roqueforti* are eukaryotic microorganisms: yeast and mold, respectively. Even if the bacteria species are most frequently related to the quality and safety of the foods, they are also involved in the spoilage of refrigerated foods, thank also to their ability to grow at low temperatures (Rico-Munoz et al., 2019; Tuffi et al., 2012).

The experimentation was done testing *B. subtilis*, *E. coli*, *S. cerevisiae*, and *P. roqueforti* at roomT (22.8 ± 1.1 °C – 97 ± 3% RH), and all the six microorganisms at refT (5.4 ± 0.7 °C – 98 ± 1% RH). At both temperatures, the times of exposure were 1, 2, 3, 4, 5, and 6 h. The refT was included since it is the temperature commonly used in the food sector for

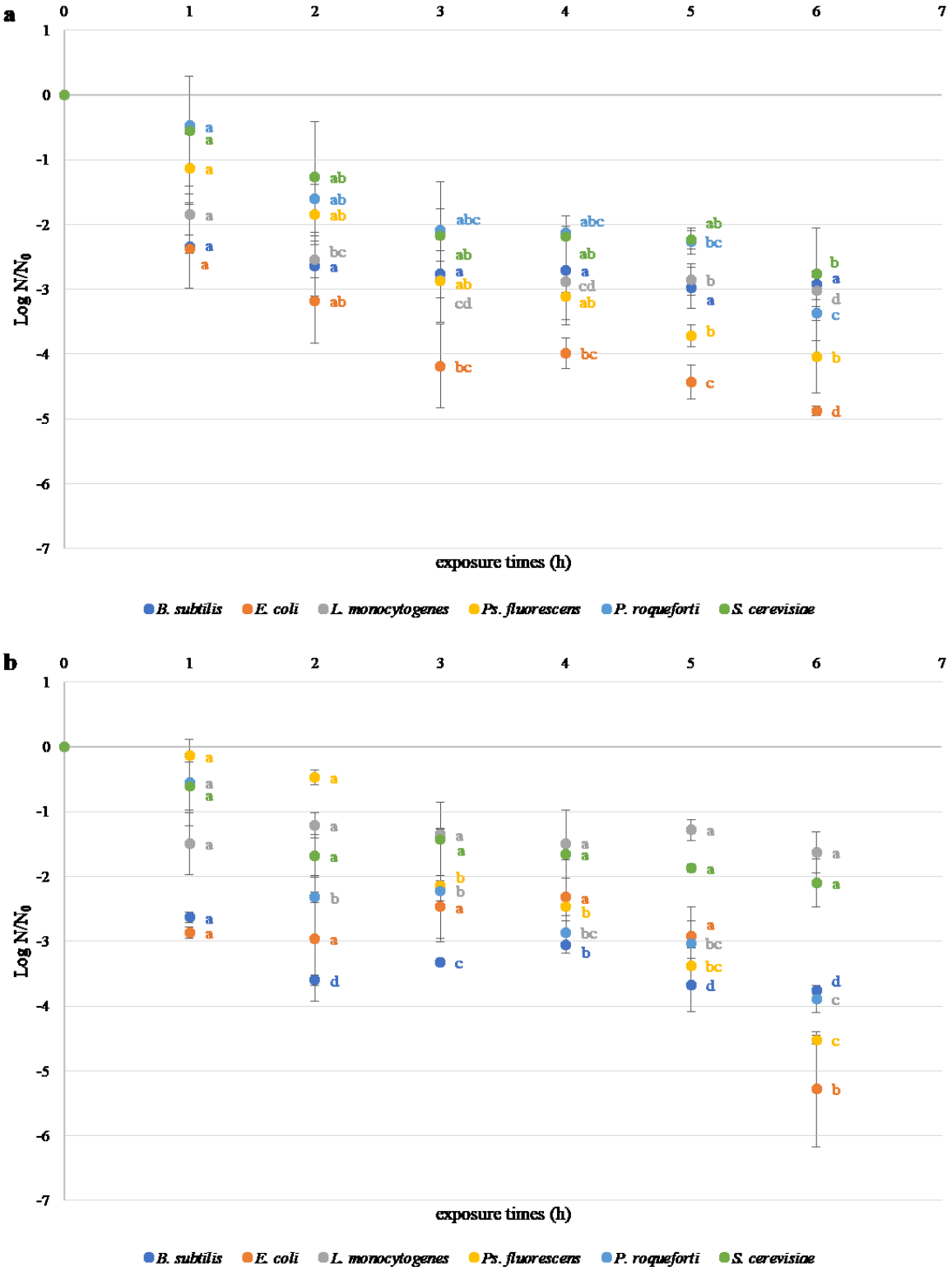

Figure 6 Effect of the treatments with DA ($4x10^6$ NAI/cm^3s + 2.20 mg/h O_3) on *B. subtilis*, *E. coli*, *L. monocytogenes*, *Ps. fluorescens*, *P. roqueforti*, and *S. cerevisiae* at roomT (22.8 ± 1.1 °C – 97 ± 3% RH) (a), and refT (5.4 ± 0.7 °C – 98 ± 1% RH) (b). Data are expressed as Log reduction (mean ± SD).

the storage of food, especially for the perishable foods that need the maintenance of the cold chain (Kampmann et al., 2009; Panou et al., 2021). Indeed, the final aim was to investigate if the ionization technology could be applied during refrigerated storage (Cárdenas et al., 2011; Muhlisin et al., 2016; Sheelamary & Muthukumar, 2011).

In general, the effect of desiccation was never observed, and the vital count of the control samples remained always 10^8-10^9 CFU/mL. The controls exposed to refT were not significantly affected by the refrigeration temperature compared with the same controls at roomT. Figure 6 illustrates the results of the treatment with DA at roomT (a) and refT (b). The results of *L. monocytogenes* and *Ps. fluorescens* at roomT obtained in experiment 1 were also reported. The antimicrobial effect of the exposition to $4x10^6$ NAI/cm^3s + 2.20 mg/h O_3 at roomT was confirmed also for the additional four species involved. The greatest effect was observed for *E. coli* with a decrease in the vitality of 4.88 ± 0.07 Log after 6 h. For the longest periods of treatment, *E. coli* and *Ps. fluorescens* were the two species with the highest effect of the ionization; this behavior can be attributed to their Gram-negative nature, for which was observed a higher sensitivity of ozone (Restaino et al., 1995). These two microorganisms were widely investigated for the evaluation of the antimicrobial effect of ionization technology (L. Fan et al., 2002; Park et al., 2016; Tyagi et al., 2008) showing be sensitive to the presence of NAI and ozone. The same similarity was observed for *B. subtilis* and *L. monocytogenes*, two Gram-positive bacteria, for which the final counts were 2.92 ± 0.87 and 3.02 ± 0.14 Log respectively. At roomT, the lower effect during the periods of exposure was observed for *P. roqueforti* and *S. cerevisiae*, both belonging to the fungi kingdom, reaching values of Log reduction of 3.37 ± 0.11 and 2.76 ± 0 respectively. At refT, the trends were quite different. For *L. monocytogenes* the reductions at refT were lower more than 1 Log in all the exposition times compared to the values at roomT. Also for *Ps. fluorescens*, in the first 2 h of treatment, the vitality remained almost unchanged. The behavior of these two microorganisms was probably due to their psychotropic nature, making an antimicrobial effect more difficult at refT. However, while the final count of *L. monocytogenes* was 1.63 ± 0.32 Log, that of *Ps. fluorescens* was significantly ($p < 0.05$) higher (4.52 ± 0.07 Log). *B. subtilis* and *E. coli* demonstrated to be quite sensitive to exposure since the first hour of treatment, with a significant ($p < 0.05$) increase of the cell death rate until the 6 h of exposure (3.75 ± 0.07 Log and 5.28 ± 0.89 Log, respectively). *P. roqueforti* and *S. cerevisiae* showed to be

affected by NAI and ozone also at refT, with the highest effect for the mold (3.89 ± 0.21 Log reductions) than the yeast (2.10 ± 0.37 Log reduction) at 6 h.

The synergism of the concentrations of NAI ($4x10^6$ NAI/cm^3s) and ozone (2.20 mg/h) generated by DA resulted to be a valid technology for the reduction of microbial vitality of the different species tested. However, the concentration of ozone generated by this device was high considering a possible application inside a domestic refrigerator. It is known that ozone toxicity is an important parameter to consider during treatments. The Federal Occupational Safety and Health Administration (OSHA, 1994) in the USA and the Health and Safety Executive (Health and Safety Executive, 2014) in the UK specify 0.2 mg/m^3 the current permissible exposure limit for continuous exposure during 8 h day/40 h week period or 0.6 mg/m^3 for a 15 min period not to be exceeded more than four times per day. The value measured inside the chamber after 6 h in setup 2 was 6.88 ± 0.30 mg/m^3 exceeding the permissible value, even if many recent works were carried out using much higher concentrations of ozone (C. Chen et al., 2020; Miller et al., 2021; Panou et al., 2021). However, in this case, the interest to investigate other devices that generate NAI and lower amounts of ozone was considered. Indeed, the aim was to investigate if the ionization technology could be applied during refrigerated storage in a domestic context avoiding high levels of ozone for consumer safety and food quality (Cárdenas et al., 2011; Muhlisin et al., 2016; Sheelamary & Muthukumar, 2011).

2.3.4 Comparison of the antimicrobial effect of DA and DM at room and refrigeration temperature (experiment 3)

A third ionizer, DM, was selected by considering its different properties of generation of NAI and ozone ($1x10^7$ NAI/cm^3s + 0.02 mg/h O_3) compared to DA ($4x10^6$ NAI/cm^3s + 2.20 mg/h O_3). Since experiment 1 showed that different concentrations of NAI and ozone led to different antimicrobial effects on *L. monocytogenes* and *Ps. fluorescens*, to explore the potentiality of this device the same test was carried out. *L. monocytogenes* and *Ps. fluorescens* were exposed to ionization treatment with DM for 1, 2, 3, 4, 5, and 6 h at roomT and refT, and the results were compared to those obtained with DA for the same test. To facilitate comparison, also the results of *L. monocytogenes* and *Ps. fluorescens* at ref T reported in Figure 6b were shown in Table 10. The effect of desiccation was never observed and the vital count of the control samples remained always 10^8-10^9 CFU/mL.

Moreover, the controls exposed to refT were not significantly affected by the refrigeration temperature compared with the same controls at roomT, underlying the psychrotrophic nature and adaptation of the two microorganisms to low temperature (Collins & Margesin, 2019). The exposition to ionization treatment under low concentration of ozone was effective for the reduction of *L. monocytogenes* at roomT and refT, but any effect was not observed for *Ps. fluorescens* neither at roomT nor at refT. For both microorganisms, the efficacy of the treatment was observed to be higher using DA ($4x10^6$ NAI/cm^3s + 2.20 mg/h O_3), bringing out what was previously found by other studies regarding the importance of the concentrations of NAI and ozone (L. Fan et al., 2002; Fletcher et al., 2007). Focusing on *L. monocytogenes* the antimicrobial effect after 6 h was higher at roomT (reductions of 3.03 ± 0.14 Log with DA and 2.42 ± 0.11 Log with DM) than refT (reductions of 1.63 ± 0.32 Log with DA and 1.34 ± 0.72 Log with DM) independently from the amount of NAI or ozone generated by the devices. This behavior could find a possible explanation in the psychrotrophic nature of this microorganism, for which the ability to adapt to cold stress conditions through the production of cold shock proteins is demonstrated (Santos et al., 2019). Effectively, at refT with both devices, after the reduction during the first hour of exposure, there was not observed any further significant reduction and the trend remained similar until the 6 h of the treatment. Similar behavior was previously found out by Marino et al. (2018) after the exposition of microbial biofilms to ozonated water and gaseous ozone, suggesting that the antimicrobial effect of these oxidative technologies (i.e. NAI and ozone) can be achieved in the first stages of the exposition. Another observation was that the exposition of 6 h at refT produced a similar result for both conditions identified (reductions of 1.63 ± 0.03 Log with DA and 1.34 ± 0.11 Log with DM) suggesting that during the exposures for prolonged periods, NAI and low concentration of ozone can achieve similar results that in the presence of NAI and high concentration of ozone. A study by Boumail et al. (2016) demonstrated the antimicrobial effect of negative air ionization with a minimal amount of ozone on ready-to-eat cauliflower florets inoculated with *L. innocua*, showing a reduction of 2 Log CFU/g after an exposition of one week to NAI treatment. These findings suggested the possibility to increase the period of the treatment to avoid the use of dangerous levels of ozone, which is toxic for the human at certain conditions of exposition (Karaca & Velioglu, 2007), for the treatment and conservation of food at refT in the food sector.

Table 10 Comparison of the treatments with DA ($4x10^6$ NAI/cm^3s + 2.20 mg/h O_3) and DM ($1x10^7$ NAI/cm^3s + 0.02 mg/h O_3) *on L. monocytogenes* and *Ps. fluorescens* at roomT (22.8 ± 1.1 °C – 97 ± 3% RH) and refT (5.4 ± 0.7 °C – 98 ± 1% RH). Data are expressed as Log reduction (mean ± SD).

	L. monocytogenes				***Ps. fluorescens***			
Exposure times (h)	**DA**		**DM**		**DA**		**DM**	
	roomT	refT	roomT	refT	roomT	refT	roomT	refT
1	1.85 ± 0.32 $^{a\,B}$*	1.49 ± 0.48 $^{a\,A}$	0.32 ± 0.52 $^{a\,A}$	0.41 ± 0.45 $^{a\,A}$	1.13 ± 0.87 $^{a\,A}$	0.14 ± 0.02 $^{a\,A}$	0.03 ± 0.04 $^{a\,A}$	0.03 ± 0.03 $^{a\,A}$
2	2.54 ± 0.28 $^{bc\,A}$	1.21 ± 0.19 $^{a\,A}$	1.23 ± 0.53 $^{a\,A}$	1.21 ± 0.49 $^{a\,A}$	1.84 ± 0.47 $^{ab\,A}$	0.47 ± 0.11 $^{a\,A}$	0.08 ± 0.08 $^{a\,A}$	0.02 ± 0.19 $^{a\,A}$
3	2.86 ± 0.04 $^{cd\,B}$	1.35 ± 0.09 $^{a\,A}$	1.14 ± 0.21 $^{a\,A}$	0.98 ± 0.06 $^{a\,A}$	2.87 ± 0.64 $^{ab\,A}$	2.14 ± 0.87 $^{b\,A}$	0.26 ± 0.22 $^{a\,A}$	0.16 ± 0.24 $^{a\,A}$
4	2.88 ± 0.17 $^{cd\,B}$	1.50 ± 0.53 $^{a\,A}$	1.65 ± 0.56 $^{a\,A}$	1.57 ± 0.78 $^{a\,A}$	3.21 ± 0.36 $^{ab\,A}$	2.46 ± 0.72 $^{b\,A}$	0.40 ± 0.71 $^{a\,A}$	0.45 ± 0.54 $^{a\,A}$
5	2.85 ± 0.24 $^{b\,B}$	1.28 ± 0.16 $^{a\,A}$	1.28 ± 0.73 $^{a\,A}$	1.18 ± 0.76 $^{a\,A}$	3.72 ± 0.17 $^{b\,A}$	3.38 ± 0.70 $^{bc\,A}$	0.00 ± 0.05 $^{a\,A}$	0.05 ± 0.03 $^{a\,A}$
6	3.03 ± 0.14 $^{d\,B}$	1.63 ± 0.32 $^{a\,A}$	2.42 ± 0.11 $^{a\,A}$	1.34 ± 0.72 $^{a\,A}$	4.04 ± 0.56 $^{b\,A}$	4.52 ± 0.07 $^{c\,A}$	0.06 ± 0.17 $^{a\,A}$	0.04 ± 0.19 $^{a\,A}$

*For each microorganism and then ionizer, means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$); means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

The results of *Ps. fluorescens* showed a different trend of microbial reduction compared to that of *L. monocytogenes*. The difference between the efficacy related to the presence of different amounts of ozone resulted very clear, with DM ($1x10^7$ NAI/cm^3s + 0.02 mg/h O_3) not being able to generate any logarithmic reduction in the test conditions adopted in the study. Probably, the presence of NAI and low concentration of ozone (ozone was not detected inside the chamber in the presence of inoculated plates during the characterization of the devices) exerted a sort of sub-lethal response by this bacteria, which can adapt its metabolism to survive oxidative stress (Mailloux et al., 2011). Moreover, this behavior may be related to the different structure and composition of the cell wall of Gram-negative than Gram-positive bacteria. Differently from that reported by Restaino et al. (1995), Pascual et al. (2007) stated that Gram-positive bacteria are more sensitive than Gram-negative to the exposition of ozone, and therefore its very low presence using DM condition can explain the sensitivity of *L. monocytogenes* but the non-effect on *Ps. fluorescens*. However, the sensitivity to ozone can be different for each microorganism (Marino et al., 2018), and also two Gram-negative bacteria could have different behavior in the presence of this molecule. On the contrary, DA ($4x10^6$ NAI/cm^3s + 2.20 mg/h O_3) caused a reduction in viability at both temperatures of exposition, alluding that the presence of ozone at high concentration was essential to achieve the microbial death of this microorganism. This observation was in agreement with the results by Fan et al. (2002), who found that the exposure of NAI alone did not affect the viability of *Ps. fluorescens*, but the simultaneous exposure to ozone and NAI caused an important rate of cell death. Considering the exposition of *Ps. fluorescens* to NAI and ozone at 2.20 mg/h, the antimicrobial effect appeared to be slower at refT than roomT in the first three hours of the treatment, probably for the psychrotrophic nature of this microorganism. The effect of the exposition became significantly greater ($p < 0.05$) after 6 h of exposure time, generating reductions of 4.04 ± 0.56 Log at roomT and 4.52 ± 0.07 Log at refT. By the comparison between DA and DM emerged that the presence of a high concentration of ozone improved the antimicrobial effect of NAI in the short treatment times. However, even if slowly, also the low concentration of DM (0.02 mg/h) allowed to achieve an antimicrobial effect for *L. monocytogenes.* Thanks to the low amount of ozone, this device could be safely used inside a domestic refrigerator; therefore, an interest to investigate if the action of NAI towards microorganisms can be enhanced by a continuous and protracted exposition come out.

2.3.5 Antimicrobial effect of DM at refrigeration temperature for extended periods (experiment 4)

B. subtilis, *E. coli*, *L. monocytogenes*, *Ps. fluorescens*, *P. roqueforti*, and *S. cerevisiae* were exposed to ionization treatment using DM ($1x10^7$ NAI/cm^3s + 0.02 mg/h) for 4, 16, 24, and 72 h at refT (Figure 7). The only refT was considered for this part of the study since the final aim was the possible application of the ionization technology inside domestic refrigerators for improving the quality of air and surfaces.

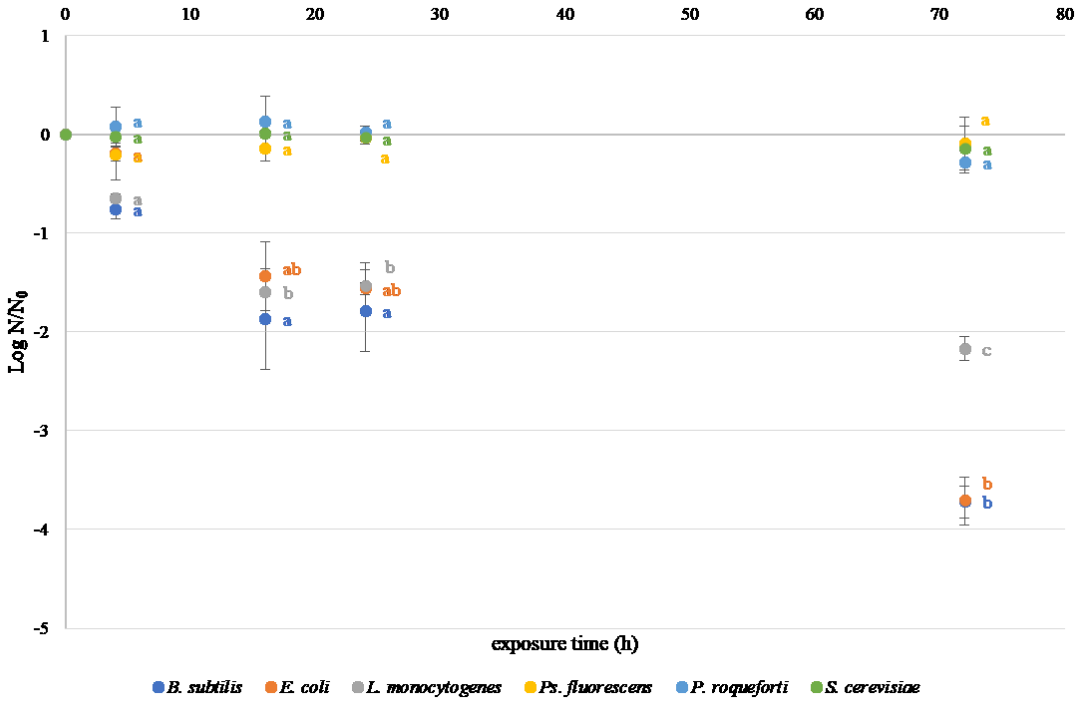

Figure 7 Effect of the treatments with DM ($1x10^7$ NAI/cm^3s + 0.02 mg/h) on *B. subtilis*, *E. coli*, *L. monocytogenes*, *Ps. fluorescens*, *P. roqueforti*, and *S. cerevisiae* at refT (5.4 ± 0.7 °C – 98 ± 1% RH). Data are expressed as Log reduction (mean ± SD).

The exposure times were extended to 72 h since the amount of ozone was very low (0.02 mg/h) and the effect of the synergism between NAI and ozone could be appreciated by prolonging the time of the treatment. As in the previous assessments, a desiccating effect was not observed and the vital count of the control samples remained always 10^8-10^9 CFU/mL; the controls exposed to refT were not significantly affected by the refrigeration temperature compared with the same controls at roomT. For *L. monocytogenes* was confirmed the antimicrobial effect of the exposition to NAI with a low amount of ozone: after 16 h of the exposition the decrease of the viability was significant ($p < 0.05$) reaching

1.59 ± 0.01 Log reductions. Increasing the exposition time to 24 h, an almost unchanged effect was observed, and extending the ionization treatment to 72 h a further effect on microbial vitality was obtained, leading to a final reduction of 2.17 ± 0.12 Log. A similar result was obtained by Arnold et al. (2004), who have reached 3.70 Log reductions after 3 h of biofilm treatment with ESCS (Electrostatic Space Charge System), a technology designed to produce NAI minimizing the contextual generation of ozone. *B. subtilis* and *E. coli* resulted in the microorganisms with the highest effect of the treatment, reaching nearly 4 Log reductions after 72 h of exposure. These results are in agreement with Arnold et al. (2004) and Park et al.(2016) confirming the antimicrobial potential of NAI. Particularly, Park et al. (2016) have verified by an ion capture system that the only ozone generated had very little or no bactericidal effect, demonstrating the great contribution of NAI in the killing of the bacteria. Moreover, L. Fan et al. (2002), despite using a low concentration of ozone (100 nL/L), found that the synergism between ozone and NAI was indispensable to achieve the death of the bacterial cells of *E. coli* and *Ps. fluorescens* with the degree of the effect depending on the microorganism. Nevertheless, neither in this part of the experimentation, an effect was observed for *Ps. fluorescens* by extending the exposure to 72 h confirming that for this microorganism the contribution of high levels of ozone was central to exert an antimicrobial effect. Also for the yeast and the mold, a significant effect was not observed after 72 h of exposure, even if a fungistatic effect was previously verified for *Botrytis cinerea* and *Penicillium expansum* (Chauhan et al., 2015).

2.4 Conclusions

Ionization technology is considered a rapid, green, and low-cost strategy. Its antimicrobial effect was evaluated on six food-related microorganisms having different qualities in terms of intrinsic characteristics and impact in the food sector. Generally, this technology turned out to be able to affect microbial viability and the range of the antimicrobial effect was dependent on several factors, like the amounts of NAI and ozone generated and their synergism, the specific microorganism, the temperature, and the time of the exposition to the treatment. The evidence of the synergism between NAI and ozone was confirmed also when low levels of ozone were present. This outcome was very important especially considering the possibility to use this technology in total safety also in a domestic environment concerning the concentration of ozone present during the treatments. Therefore, the

correct application of operative conditions (i.e., increase the period of the treatments in conditions of low levels of ozone) resulted be important to achieve a significant reduction of microbial vitality, especially in the case of dangerous microorganisms as the pathogenic *L. monocytogenes*.

PART 2

IONIZATION TECHNOLOGY FOR THE APPLICATION INSIDE THE DOMESTIC REFRIGERATOR

The antimicrobial effect of ionization technology observed on the model systems gave promising results, underling the ability of NAI and ozone to affect the vitality of the microorganisms. To have the possibility to use this technology during the cold storage for improving the hygienic status of the environment in which the food is stored, the interest was to understand what the effect of this technology on food could be. For that purpose, DA and DM, which generated NAI and different amounts of ozone were tested on different food categories, i.e., vegetables, fruits, and mushrooms. The effect of the presence of oxidative active species, as NAI and ozone, and their interaction with food components has been evaluated through the changes in the color, while the influence on the natural microflora of the examined food has been done through the microbiological traditional count of the viable cells.

3 STUDY OF DIFFERENT NAI AND OZONE CONDITIONS OF IONIZATION TECHNOLOGY ON FOOD

3.1 Introduction and aim of the study

Fruits, vegetables, and mushrooms are foods widely produced and consumed all over the world (Del Río-Celestino & Font, 2020). They are considered fresh foods, therefore appropriate storage after harvest is required for the maintenance of their fresh-like characteristics. In recent years, research has investigated numerous technologies with the aim to guarantee not only a safe product from the microbiological point of view but also to preserve the food's appearance, physicochemical and nutritional properties. These so-called novels non-thermal technologies are high-hydrostatic pressures, pulsed electric fields, ultrasound, ultraviolet light, pulsed light, and cold plasma (Morales-de la Peña et al., 2019). Among these technologies, also ionization can be included, thanks to the similarity with non-thermal plasma (Muhammad et al., 2018). The studies of the food treatments with ionization technology are still few (see section 1.6), but its antimicrobial effect was certainly more studied and recognized (Digel et al., 2005; L. Fan et al., 2002; Fletcher et al., 2007; Tyagi et al., 2008). Considering these premises and the previous results obtained with the ionization treatment on the model systems, the ionization technology of DA and DM was used to carry out an explorative investigation on the treatment of foods.

This study aimed to assess the effect of NAI and different amounts of ozone on vegetables, berry fruits, and mushrooms through colorimetric and microbiological analysis.

3.2 Materials and methods

3.2.1 Preparation of the treatment chamber

The same 0.045 m^3 air-tight chamber used for the model systems tests (section 2.2.5) was used for the assessment of ionization technology on food.

3.2.2 Exposition of food to ionization treatment

Lettuce (*Lactuca sativa* var. *crispa*), endive (*Cichorium endiva*), zucchini (*Cucurbita pepo*), tomato (*Solanum lycopersicum*), strawberry (*Fragaria x ananassa*), blueberry (*Vaccinium myrtillus*), and mushroom (*Agaricus bisporus*) were purchased from a local market (Porcia, PN, Italy) and were stored at 4.7 ± 0.3 °C at 74 ± 13% RH for 2 h under plastic bags until the treatment to prevent any modification of the food before the test. Before placing the foods in the chamber, leaves with discoloration, spots, or defects were taken off from lettuce and endive; zucchini, tomatoes, strawberries, and blueberries with defects were removed; soil residuals were taken off from mushrooms. Finally, the most possible homogeneous food samples were placed inside the chamber and used for testing. The foods were exposed to the treatment at refrigeration temperature (nominal value setting: 4 ± 1 °C). The control foods were put inside an identical air-tight chamber at refrigeration temperature without the presence of the ionizer.

The exposition on food to ionization treatment was divided into two parts.

Part 1. The first part aimed to perform an explorative assessment on the food of the effect of the different concentrations of NAI and ozone generated by DA ($4x10^6$ NAI/cm^3s + 2.20 mg/h O_3) and DM ($1x10^7$ NAI/cm^3s + 0.02 mg/h O_3). The investigation was done using lettuce, strawberry, and mushroom because these foods were reported in the company's internal protocol for the evaluation of the shelf-life of fresh products. The protocol specified the procedures for the evaluation of the food storage performances of the refrigerator drawers intended for the preservation of fruits and vegetables. The exposure of food to the treatment was 4 days according to the following working modalities of the ionizers:

- DA: 5 min ON (generation of NAI and ozone) – 3 h OFF (stop of NAI and ozone generation) cycle;
- DM: continuous work.

Part 2. Considering the results obtained in part 1, DM ($1x10^7$ NAI/cm^3s + 0.02 mg/h O_3) was selected to carry out further tests increasing the period of exposure and including additional fruits and vegetables. Therefore, lettuce, endive, zucchini, tomato, blueberry, and mushroom were exposed to ionization treatment for 9 days using DM in the continuous working modality.

3.2.3 Visual monitoring of the foods

For monitoring the changes of the food during the exposition to the treatment, photos were taken using a compact camera with 20 x optical zoom (Sony, DSC-WX350 model).

3.2.4 Color determination

A portion of lettuce, mushroom, and strawberry was kept from the treatment and reference boxes at days 0, 2, and 4 (part 1) or days 0, 3, 7, and 9 (part 2) and immediately subjected to analysis. Color parameters measurements were performed using a portable spectrophotometer (x-rite SP64, Grand Rapids, Michigan, USA). For each food sample, 10 points of reading were identified by hitting the surface as homogeneously as possible, trying to standardize the color measurement Figure 8. The results were expressed as the average of the measurements.

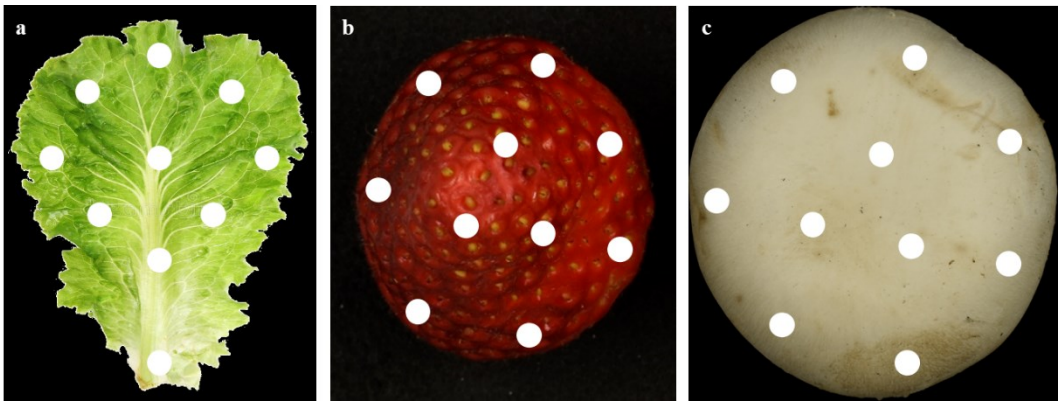

Figure 8 Identification of 10 points of reading for lettuce leaf (a), strawberry whole fruit (b), and mushroom cap (c) for color measurements with the portable spectrophotometer.

Color changes were quantified in the CIE $L^*a^*b^*$ color space. The instrument was calibrated using a white and a black tile. L^* parameter refers to the lightness and ranges from 0 (black) to 100 (white); a^* parameter measures the degree of redness ($+a^*$) or greenness ($-a^*$); b^* parameter measures the degree of yellowness ($+b^*$) or blueness (-

b^*). From the CIE L^*, a^*, b^* parameters, Chroma (C^*, which refers to the color saturation), the Hue angle ($H°$, which describes the hue of an object), and total color difference (ΔE^*) were calculated. For mushrooms, also the browning index (*BI)* was calculated (Vallespir et al., 2019). The formulas were the following:

1 $$H° = arctan\frac{b^*}{a^*}, when\ a^* and\ b^* were > 0$$

2 $$H° = 180 + arctan\frac{b^*}{a^*}, when\ a^* was < 0\ and\ b^* was > 0$$

3 $$H° = 360 + arctan\frac{b^*}{a^*}, when\ a^* was > 0\ and\ \ b^* was < 0$$

4 $$C^* = \sqrt{a^{*2} + b^{*2}}$$

5 $$\Delta E = \sqrt{(L_n^* - L_0^*)^2 + (a_n^* - a_0^*)^2 + (b_n^* - a_0^*)^2}$$

where:

L^*_0 = luminosity at day 0 and L^*_n = luminosity at days 2, 3, 4, 7 and 9

a^{*0} = a* value at day 0 and a^{*n} = a* value at days 2, 3, 4, 7 and 9

b^*_0 = b* value at day 0 and b^*_n = b* value at days 2, 3, 4, 7 and 9

6 $$BI = \frac{browning_{day\ n}}{brownin_{day\ 0}}$$

$$Browning = 100 \times \left(\frac{X - 0.31}{0.71}\right)$$

$$X = \frac{a^* + 1.75L^*}{5.645L^* + a^* - 3.012b^*}$$

3.2.5 General aspects of the color parameters

Mokrzycki & Tatol (2011) made a survey in which they identified that a standard observer sees the difference in color according to the following ranges:

- $0 < \Delta E^* < 1$ observer does not notice the difference
- $1 < \Delta E^* < 2$ only experienced observers can notice the difference
- $2 < \Delta E^* < 3.5$ unexperienced observers also notice the difference
- $3.5 < \Delta E^* < 5$ clear difference in color is noticed
- $5 < \Delta E^*$ observer notice two different colors.

ΔE^* value describes the total color difference, including the changes in lightness (L^*), in redness or greenness (a^*), and in yellowness or blueness (b^*) From the a^* and b^* values, $H°$ and C^* were calculated. $H°$ is an angular measurement that describes the hue angle, C^* is referred to the intensity of the color (C* values near to 0 indicate a

greater presence of grey) (Figure 9). CIE *L*a*b** uses Cartesian coordinates to calculate a color in a color space while CIE *L*C*H°* uses polar coordinates. This consent to increase the % of agreement with the visual observation from 75% of CIE *L*a*b** to 85% of CIE *L*C*H°* (this information was reported in the guide to understanding color communication of the colorimeter X rite).

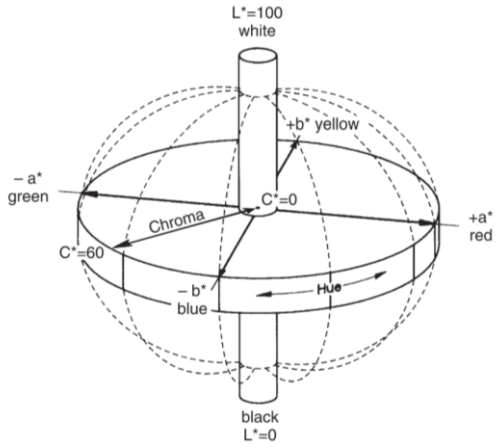

Figure 9 System representing the color parameters (from X rite color guide).

3.2.6 Microbiological analysis

Microbiological analysis was carried out at days 0, 2, and 4 (part 1) or days 0, 3, 7, and 9 (part 2) by transferring a portion of lettuce, mushroom, and strawberry in a stomacher bag (Stomacher® 400 Classic bags with filter), containing 9 parts (w:v) of Maximum Recovery Diluent (MRD, Oxoid, Milan, Italy), and homogenized for 2 min at 250 strokes per min using a stomacher (Star-Blender digital, VWR). Decimal serial dilutions were done and plated on Plate Count Agar (PCA, Oxoid, Milan, Italy) for the total bacterial count. Plates were incubated at 30 °C for 24-48 h.

3.2.7 Statistical analysis

Each trial was carried out in at least two biological replicates and the data were expressed as the mean ± standard deviation (SD). Analysis of variance (ANOVA) was performed by using Statistics 8.0 (Statsoft software, Tulsa, Oklahoma, USA). Differences between the means were assessed using Tukey's HSD post-hoc test.

3.3 Results and discussion

3.3.1 Ionization treatment on lettuce, strawberry, and mushroom using DA and DM (part 1)

Lettuce, mushroom, and strawberry were exposed to ionization treatment for 4 days with DA ($4x10^6$ NAI/cm^3s + 2.20 mg/h O_3) and DM ($1x10^7$ NAI/cm^3s + 0.02 mg/h O_3). DA was set to work according to a cycle mode, with 5 min of NAI and ozone generation and 3 h of pause. This modality was established because of the high production of ozone and therefore the possibility to observe oxidative damages on the foods if the working mode would have been continuous (Ayranci et al., 2020; Fundo et al., 2018). The appearance of the food represents one of the most important characteristics that lead the consumers to decide on what to do with the food they are watching (eat, cook or throw away) (Panou et al., 2021). For that purpose, together with the colorimetric analysis, the monitoring of visual observation of the foods was carried out through the collection of photos during the storage period to detect any modifications of the products with the naked eye. The photos were taken using a compact camera at the opening of the chambers when the foods were sampled for the colorimetric and microbiological analysis at days 0, 2, and 4; at days 1 and 3, the chamber was opened specifically for taking the photos. An overview of the treatments performed on lettuce, strawberry, and mushroom was showed in Table 11 (DA) and Table 12 (DM). With the treatment with $4x10^6$ NAI/cm^3s + 2.20 mg/h O_3 generated by DA, it was possible to appreciate the difference of foods between control and treatment chambers starting from day 2. The alteration was most evident for lettuce and this consideration remained similar until the end of the test. For mushroom and strawberry, the difference between control and treated samples was more difficult to appreciate looking at the photos, but the visual observation allowed to identify a slight browning of treated mushroom and strawberry. During the treatment with $1x10^7$ NAI/cm^3s + 0.02 mg/h O_3 (Table 12) any visual difference between control and treated lettuce was not observed during the 4 days of exposure to NAI and ozone. However, from day 2 the treated mushroom appeared to be slightly browned, and the trend of browning increased gradually until the end of the test.

The values of temperature and relative humidity were monitored during the test. The temperature of the refrigerator was set at a nominal value of 4 ± 1°C as suggested by EFSA Panel on Biological Hazards (Koutsoumanis et al., 2020) and Food and Drug Administration FDA (FDA, 2021). The checked values were always higher, with a minimum of 6.5 ± 0.2 °C and a maximum of 6.7 ± 0.1 °C during the test with the DA, and a minimum of 6.1 ± 0.3 °C and a maximum of 7.2 ± 0.3 °C during the test with the DM. James et al. (2008) reviewed the performances and the use of domestic refrigerators in the world showing that the mean temperature ranged from −1 °C to 11 °C and the overall mean temperature was 6 °C, with 70% of refrigerators operating at average temperatures above 5 °C. Therefore, the temperatures monitored in the present study can represent a common situation in which lettuce, strawberry, and mushroom are stored in a domestic environment. The relative humidity was very high in both the treatments with DA and DM, reaching values of 100% RH in control and treatment chambers. Similar conditions were found by J. Song et al. (2000) in their study of ionization on onions, considering this value a serious problem related also to the presence of mold in the storage room used for the experimentation. In addition, condensed water was always present inside the chambers. This outcome was probably due to a combined effect of the transpiration rate of the food samples, which is directly related to temperature, and the air-tightness of the chambers. In general, the evaporation of moisture from fresh fruit and vegetables occurs simultaneously with heat removal and the fresh produce dehydration occurs gradually due to food moisture evaporation (Laguerre & Flick, 2010; Volpe et al., 2018). Lettuce, strawberry, and mushroom are foods with a very high content of water (94.7 g, 91.8 g, and 91.1 g on 100 g of fresh weight, respectively) (USDA, 2019a, 2019b, 2021), and the respiration inside a tightness chamber that did not allow the leak of the water and the exchange of the air with the outside air of the refrigerator caused the reaching of the dew point and the consequent formation of condensation. Considering the ranges of temperature and relative humidity reported in the datasheets of the devices (Table 7, section 2.2.4), the temperature monitored in the food tests was within the reported values, but the relative humidity was higher than the recommended range. However, it should be considered that the conditions of the air-tight chamber in which the treatments were carried out did not represent a real situation of fruits and vegetable storage inside the drawer of a

refrigerator. The decision to conduct the study in these operative conditions was made since the chamber represented a controlled situation in which all the active species generated by the device remained inside the chamber interacting with the foods without leakage. The visual observation monitored through the photos allowed to conclude that different concentrations of NAI and ozone led to different responses by the foods. Lettuce was subjected to oxidation phenomena during the treatment with $4x10^6$ NAI/cm^3s + 2.20 mg/h O_3 but it was not affected by the use of $1x10^7$ NAI/cm^3s + 0.02 mg/h O_3 suggesting that for this food the concentration of ozone is a decisive factor during the storage under treatment conditions. On the contrary, mushrooms appeared more browned during the treatment with DM than DA, and strawberries seemed to be not greatly affected by the high concentration of ozone.

Table 11 Overview of the ionization treatment on lettuce, mushroom, and strawberry with DA ($4x10^6$ NAI/cm^3s + 2.20 mg/h O_3) in the cycle working mode (5 min ON – 3 h OFF).

Day	Control chamber	Treatment chamber	T/RH values	Observations
Day 0			6.5 ± 0.2 °C 100% RH	-
Day 1			6.6 ± 0.1 °C 100% RH	Slight browning of mushrooms and lettuce, especially in the treatment chamber. Any visual modification not observed in strawberry.
Day 2			6.7 ± 0.1 °C 100% RH	The browning seemed to be higher than day 1 in mushrooms and lettuce, especially in the treatment chamber. Any visual modification not observed in strawberry. Presence of condensed water in both chambers.
Day 3			6.6 ± 0.1 °C 100% RH	Very evident browning of treated lettuce. Slight browning of treated mushroom and strawberry. Presence of condensed water in both chambers.
Day 4			6.5 ± 0.1 °C 100% RH	Strong browning in treated lettuce. Slight browning of treated mushroom and strawberry. Presence of condensed water in both chambers.

Table 12 Overview of the ionization treatment on lettuce and mushroom with DM ($1x10^7$ NAI/cm^3s + 0.02 mg/h O_3) in the continuous working mode.

Day	Control chamber	Treatment chamber	T/RH values	Observations
Day 0			6.1 ± 0.3 °C 100% RH	-
Day 1			6.4 ± 0.3 °C 100% RH	Any visual modification compared to day 0. Any visual difference between foods of control and treatment chambers. Presence of condensed water in both chambers.
Day 2			7.2 ± 0.3 °C 100% RH	Any visual modification than day 1 for lettuce. Any visual difference between control and treated lettuce. Mushrooms appeared slightly browned in both chambers. Presence of condensed water in both chambers.
Day 3			6.5 ± 0.2 °C 100% RH	Any visual difference between control and treated lettuce. Treated mushrooms appeared more browned than the previous days. Presence of condensed water in both chambers.
Day 4			6.2 ± 0.5 °C 100% RH	Any visual difference between control and treated lettuce. Treated mushrooms appeared more browned than the previous days. Presence of condensed water in both chambers.

3.3.1.1 Color determinations

Food color is one of the principal characteristics of food quality, as it is related to the appearance that is the first sensorial characteristic used by the consumers for evaluating the food. For this reason, together with the subjective visual observation of the human eye, the objective determination of color parameters can be very important to understand how the effect of a treatment can influence the appreciability of food by the consumers. The color parameter can be generally included in the acceptability test of fresh food products during the sensory analysis together with other attributes like the overall visual quality or off-odors (Fasciglione et al., 2020). However, the determination of color was considered suitable for the general purpose. The results of the color parameter measurements are shown in Table 13 (lettuce),

Table **14** (strawberry), and Table 15 (mushroom). The L^* parameter, which refers to the lightness of the sample, is influenced by the surrounding luminosity at the moment of taking a photo. Being aware that the environmental conditions were not standardized and therefore they could be different every time, this parameter was not considered reliable on its own and was included in the calculation for the total color change (ΔE^*).

Table 13 Effect of ionization treatment with DA ($4x10^6$ NAI/cm^3s + 2.20 mg/h O_3) and DM ($1x10^7$ NAI/cm^3s + 0.02 mg/h O_3) on L^*, a^*, b^*, C^*, $H°$, and ΔE^* of lettuce (mean ± SD).

		DA		**DM**	
	days	control	treated	control	treated
L*	0	64.39 ± 1.63 $^{a\,A*}$	62.39 ± 1.48 $^{a\,A}$	63.44 ± 1.29 $^{a\,A}$	65.44 ± 1.79 $^{a\,A}$
	2	63.62 ± 1.51 $^{a\,A}$	59.50 ± 1.28 $^{a\,A}$	64.67 ± 1.74 $^{a\,B}$	66.59 ± 1.65 $^{a\,A}$
	4	60.31 ± 2.43 $^{a\,A}$	58.32 ± 1.76 $^{a\,A}$	64.18 ± 1.36 $^{a\,A}$	64.13 ± 1.43 $^{a\,A}$
a*	0	-5.62 ± 1.45 $^{a\,A}$	-5.24 ± 1.26 $^{a\,A}$	-5.25 ± 1.58 $^{a\,A}$	-5.15 ± 0.15 $^{a\,A}$
	2	-6.15 ± 0.98 $^{a\,A}$	-6.48 ± 1.78 $^{a\,A}$	-5.49 ± 1.68 $^{a\,A}$	-5.43 ± 1.38 $^{a\,A}$
	4	-6.67 ± 1.24 $^{a\,A}$	-6.52 ± 1.15 $^{a\,A}$	-5.89 ± 1.76 $^{a\,A}$	-5.64 ± 1.24 $^{a\,A}$
b*	0	17.71 ± 2.18 $^{c\,A}$	17.71 ± 1.76 $^{a\,A}$	15.13 ± 1.43 $^{a\,A}$	15.13 ± 1.67 $^{a\,A}$
	2	23.75 ± 1.47 $^{b\,A}$	15.10 ± 2.43 $^{a\,B}$	15.84 ± 1.87 $^{a\,A}$	16.47 ± 0.99 $^{a\,A}$
	4	26.01 ± 1.53 $^{a\,A}$	15.88 ± 2.43 $^{a\,B}$	17.44 ± 1.39 $^{a\,A}$	16.47 ± 2.43 $^{a\,A}$
C*	0	18.58 ± 1.65 $^{b\,A}$	18.47 ± 1.63 $^{a\,A}$	16.01 ± 2.45 $^{a\,A}$	15.98 ± 1.52 $^{b\,A}$
	2	24.53 ± 1.96 $^{a\,A}$	16.43 ± 0.98 $^{c\,B}$	16.76 ± 1.67 $^{a\,B}$	17.34 ± 2.21 $^{a\,A}$
	4	26.85 ± 2.28 $^{a\,A}$	17.16 ± 1.75 $^{b\,B}$	18.40 ± 1.83 $^{a\,A}$	17.41 ± 2.11 $^{a\,A}$
H°	0	178.74 ± 0.15 $^{a\,A}$	178.72 ± 0.11 $^{a\,A}$	178.76 ± 0.06 $^{a\,A}$	178.76 ± 0.09 $^{a\,A}$
	2	178.68 ± 0.23 $^{a\,A}$	178.83 ± 0.09 $^{a\,A}$	178.76 ± 0.11 $^{a\,A}$	178.75 ± 0.08 $^{a\,A}$
	4	178.68 ± 0.10 $^{a\,A}$	178.82 ± 0.06 $^{a\,A}$	178.75 ± 0.03 $^{a\,A}$	178.76 ± 0.10 $^{a\,A}$

Δ***E****	0	-	-	-	-
	2	6.11 ± 1.28 $^{b\,A}$	4.28 ± 2.12 $^{a\,A}$	1.44 ± 1.10 $^{b\,B}$	1.79 ± 1.15 $^{a\,A}$
	4	9.30 ± 2.54 $^{a\,A}$	5.23 ± 3.06 $^{a\,A}$	2.51 ± 1.23 $^{a\,A}$	1.94 ± 1.26 $^{a\,A}$

*For each color parameter and ionizer, means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$); means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Differently, the *a** and *b** parameters, which refer to colors, were considered singularly to evaluate the entity of the color changes in the foods during the treatment. Among the tested foods, *a** value increased significantly ($p < 0.05$) only for mushrooms during the treatment with DM ($1x10^7$ NAI/cm^{3}s + 0.02 mg/h O_3) indicating a browning of the sample. This result corresponded with the visual observation of the mushrooms after 4 days of the treatment with DM. In the case of the mushrooms treated with DA ($4x10^6$ NAI/cm^{3}s + 2.20 mg/h O_3), this phenomenon was not observed, probably because the high concentrations of ozone decrease the activities of the oxidases, preventing the browning of the mushroom surface (Liu et al., 2020).

Table 14 Effect of ionization treatment with DA ($4x10^6$ NAI/cm^{3}s + 2.20 mg/h O_3) on *L**, *a**, *b**, *C**, *H*°, and Δ*E** of strawberry (mean ± SD).

		DA	
	day	control	treated
L*	0	46.37 ± 2.26 $^{a\,A}$	44.13 ± 2.69 $^{a\,A}$
	2	45.99 ± 2.38 $^{a\,A}$	40.40 ± 3.37 $^{a\,A}$
	4	45.79 ± 2.14 $^{a\,A}$	42.76 ± 2.78 $^{a\,A}$
a*	0	15.01 ± 1.28 $^{b\,A}$	14.87 ± 1.94 $^{a\,A}$
	2	18.48 ± 1.76 $^{a\,A}$	16.94 ± 2.31 $^{a\,A}$
	4	18.38 ± 1.93 $^{a\,A}$	16.97 ± 2.18 $^{a\,A}$
b*	0	5.05 ± 1.64 $^{b\,A}$	5.05 ± 2.01 $^{a\,A}$
	2	7.32 ± 1.87 $^{a\,A}$	7.16 ± 1.73 $^{a\,A}$
	4	8.90 ± 1.99 $^{a\,A}$	6.87 ± 1.82 $^{a\,A}$
C*	0	15.84 ± 2.36 $^{a\,A}$	15.70 ± 3.28 $^{a\,A}$
	2	19.88 ± 2.09 $^{a\,A}$	18.39 ± 2.14 $^{a\,A}$
	4	20.42 ± 2.34 $^{a\,A}$	18.31 ± 1.63 $^{a\,A}$
H°	0	0.32 ± 0.04 $^{a\,A}$	0.33 ± 0.02 $^{a\,A}$
	2	0.38 ± 0.09 $^{a\,B}$	0.40 ± 0.07 $^{a\,A}$
	4	0.45 ± 0.06 $^{a\,A}$	0.38 ± 0.06 $^{a\,B}$
Δ***E****	0	-	-
	2	4.16 ± 1.21 $^{a\,A}$	4.76 ± 0.97 $^{a\,A}$
	4	5.15 ± 2.35 $^{a\,A}$	3.10 ± 3.12 $^{a\,A}$

*For each color parameter, means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$); means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

The b* parameter significantly increased ($p < 0.05$) for the control lettuce during the treatment with DA and control and treated mushrooms during the treatment with DM. An increase of this parameter indicated a yellowing of the samples, suggesting the significant influence of the treatment to affect the color of the products. Using *a** and *b** values, *C** and *H°* were calculated. *C** parameter decreased ($p < 0.05$) for lettuce treated with DA. The loss in color was probably due to the amount of ozone generated by device A (2.20 mg/h), which oxidized the food components, causing damages to the tissue and giving a translucent effect especially on the green part of the leaves (Ölmez & Akbas, 2009). In the case of strawberries, an increase of the *C** parameter was observed for both control and treated samples, even if it was not significant, indicating an intensification of the color saturation.

Table 15 Effect of ionization treatment with DA ($4x10^6$ NAI/cm^3s + 2.20 mg/h O_3) and DM ($1x10^7$ NAI/cm^3s + 0.02 mg/h O_3) on *L*, a*, b*, C**, *H°*, Δ*E**, and *BI* of mushroom (mean ± SD).

		DA		**DM**	
	day	control	treated	control	treated
L*	0	91.53 ± 2.38 $^{a\,A*}$	92.24 ± 3.86 $^{a\,A}$	88.96 ± 2.35 $^{a\,A}$	88.79 ± 2.35 $^{a\,A}$
	2	90.64 ± 1.45 $^{a\,A}$	85.43 ± 2.39 $^{a\,A}$	88.40 ± 2.35 $^{a\,A}$	88.02 ± 2.35 $^{a\,A}$
	4	91.76 ± 1.98 $^{a\,A}$	86.26 ± 3.12 $^{a\,A}$	75.61 ± 2.35 $^{a\,A}$	75.62 ± 2.35 $^{a\,A}$
a*	0	0.80 ± 0.35 $^{a\,A}$	1.24 ± 1.12 $^{a\,A}$	0.58 ± 1.56 $^{b\,A}$	0.51 ± 0.99 $^{b\,A}$
	2	0.9 ± 0.43 $^{a\,A}$	2.50 ± 1.54 $^{a\,A}$	1.07 ± 0.98 $^{b\,A}$	1.55 ± 1.87 $^{ab\,A}$
	4	0.18 ± 0.89 $^{a\,A}$	2.08 ± 1.45 $^{a\,A}$	5.95 ± 2.31 $^{a\,A}$	5.55 ± 2.42 $^{a\,A}$
b*	0	13.19 ± 1.37 $^{a\,A}$	13.19 ± 1.76 $^{a\,A}$	16.03 ± 1.83 $^{b\,A}$	15.50 ± 1.56 $^{b\,A}$
	2	14.70 ± 1.96 $^{a\,A}$	11.01 ± 1.47 $^{a\,B}$	18.92 ± 1.55 $^{ab\,A}$	18.48 ± 2.11 $^{ab\,A}$
	4	16.57 ± 1.65 $^{a\,A}$	11.90 ± 1.17 $^{a\,B}$	21.08 ± 2.24 $^{a\,A}$	19.23 ± 2.32 $^{a\,A}$
C*	0	13.21 ± 1.48 $^{a\,A}$	13.25 ± 0.76 $^{a\,A}$	16.04 ± 1.93 $^{a\,A}$	15.51 ± 1.87 $^{b\,A}$
	2	14.73 ± 1.94 $^{a\,A}$	11.16 ± 1.68 $^{a\,A}$	18.95 ± 2.61 $^{a\,A}$	18.54 ± 2.63 $^{ab\,A}$
	4	16.57 ± 1.81 $^{a\,A}$	12.08 ± 2.41 $^{a\,A}$	21.90 ± 3.24 $^{a\,A}$	20.01 ± 1.85 $^{a\,A}$
H°	0	1.51 ± 0.02 $^{a\,A}$	1.48 ± 0.14 $^{a\,A}$	1.53 ± 0.08 $^{a\,A}$	1.54 ± 0.13 $^{a\,A}$
	2	1.51 ± 0.09 $^{a\,A}$	1.35 ± 0.11 $^{a\,B}$	1.51 ± 0.05 $^{a\,A}$	1.49 ± 0.09 $^{a\,A}$
	4	1.56 ± 0.11 $^{a\,A}$	1.40 ± 0.21 $^{a\,A}$	1.30 ± 0.35 $^{a\,A}$	1.29 ± 0.11 $^{b\,A}$
Δ*E**	0	-	-	-	-
	2	1.76 ± 1.66 $^{a\,B}$	7.26 ± 3.41 $^{a\,A}$	2.99 ± 1.56 $^{b\,A}$	3.25 ± 1.97 $^{b\,A}$
	4	3.44 ± 1.94 $^{a\,A}$	6.17 ± 4.52 $^{a\,A}$	15.25 ± 3.14 $^{a\,A}$	18.28 ± 4.61 $^{a\,A}$
BI	0	-	-	-	-
	2	0.14 ± 0.12 $^{a\,A}$	0.01 ± 0.04 $^{a\,A}$	0.24 ± 0.23 $^{a\,A}$	0.29 ± 0.16 $^{a\,A}$
	4	0.22 ± 0.06 $^{a\,A}$	0.04 ± 0.03 $^{a\,B}$	1.06 ± 0.35 $^{a\,A}$	1.06 ± 0.29 $^{a\,A}$

*For each color parameter and ionizer, means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$); means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

For mushrooms, C^* significantly increase during the treatment with DM, reflecting the values of a^* and b^* observed in the same treatment. $H°$ parameter was not influenced in most cases suggesting that the hue did not alter during the refrigerator storage also in the presence of ionization treatment. For the three foods, the results of the total color change (ΔE^*) reflected what was observed by the naked eye during the visual assessment and were in line with the range of values proposed by Mokrzycki & Tatol (2011). For lettuce, the values were lower in the samples treated with DM than DA (1.94 and 5.23 after 4 days, respectively) indicating that the presence of low amounts of ozone did not negatively affect the food quality perception of lettuce (Sengun & Kendirci, 2018). For strawberries, ΔE^* values were similar for the control and treated samples, suggesting that the presence of ozone that is known to cause oxidative damages on food components, as anthocyanins (Tiwari et al., 2009), did not affect the color of the fruits. For mushrooms, the value of ΔE^* after 4 days was lower in the samples treated with DA (6.17 compared to 18.28 of DM). The *BI* confirmed all the results considered for mushrooms, showing that effectively after 4 days the browning of the mushrooms was higher during the tests in which NAI and a low concentration of ozone were was present. However, this outcome was true for both control and treated samples indicating that probably the problem was related to the batch used for testing mushrooms with DM. Indeed, browning after harvest is a common event in mushrooms because of enzymatic oxidation, senescence, and microbial growth causing color changes from white to brown (Lin & Sun, 2019).

The colorimetric analysis resulted to be a valid objective analysis to support the subjective naked eye visual observation of the food samples. The treatment with $4x10^6$ NAI/cm^3s + 2.20 mg/h O_3 negatively affected the quality of lettuce but it did not lead to significant changes for strawberries and mushrooms. However, the treatment with $1x10^7$ NAI/cm^3s + 0.02 mg/h O_3 affected the quality of the mushrooms. These results indicated that each food could interact differently according to different concentrations of NAI and ozone and therefore a compromise should be found to have the possibility to treat at the same time various food categories.

3.3.1.2 Microbiological analysis

The evolution of total bacterial count during the 4 days of the exposure to ionization treatment on lettuce, mushrooms, and strawberries is reported in Figure 10. The microbiological quality of the foods during the cold storage of fresh fruits, vegetables, and mushrooms represents a very important parameter to ensure food safety at the moment of consumption. For this reason, the evaluation of the trend of the microbial load during the storge was done immediately after the purchasing of the foods (day 0), and after 2 days, and at the end of the treatments (day 4).

Lettuce. The initial count of lettuce was around 6 Log CFU/g for all the heads considered in the study. This microbial load remained almost stable ($p > 0.05$) in both the control samples (DA-C and DM-C) and the treated samples with DM (DM-T) but showed an increase over the 4 days ($p < 0.05$) for the lettuce treated with the DA (DA-T). Probably, the high concentration of ozone present in the treatments with the DA (2.20 mg/h) caused important damages to the plant tissue (Koseki & Isobe, 2006) with the consequent release of nutrients that can be used by microorganisms for their growth.

Mushrooms. The microbial count of the mushrooms at day 0 ranged between 5.88 and 6.80 Log CFU/g. In all the cases evaluated, the microbial population increased significantly over time ($p < 0.05$) reaching values between 7.06 and 7.76 Log CFU/g. Fresh mushrooms are susceptible to microbial attack and represent an ideal medium for microbial growth because they have high water content and a pH near to neutrality (Brennan et al., 2000). The initial count enumerated for mushrooms was similar to those found by Akata et al. (2015), however, they obtained 2.44 and 3.07 Log reductions after 60 min of treatment at 2.8 and 5.3 mg/L of gaseous ozone, respectively, while in these treatments any reduction was observed.

Strawberries. The initial microbial count of strawberries was the same for the control and treated strawberries (5.50 Log CFU/g), but the trend during the 4 days was different for the samples. From day 2 the growth in the treated samples was significantly lower ($p < 0.05$) than the control samples (3.70 ± 0.63 Log compared to 5.01 ± 0.31 Log at day 2, 5.61 ± 0.46 Log compared to 6.30 ± 0.45 Log at day 4). This result suggested the possibility of ionization treatment to keep controlled the microbial load of strawberries in the presence of $4x10^6$ NAI/cm^3s + 2.20 mg/h O_3 (DA). Zhou et al.

(2018) have treated strawberries with lower concentrations of ozone (0.08 mg/m^3) obtaining good values of microbial reduction: their results were a valid reason to test the strawberries in the following steps of the study even in the conditions of 1x10^7 NAI/cm^{3}s + 0.02 mg/h O_3 generated by DM.

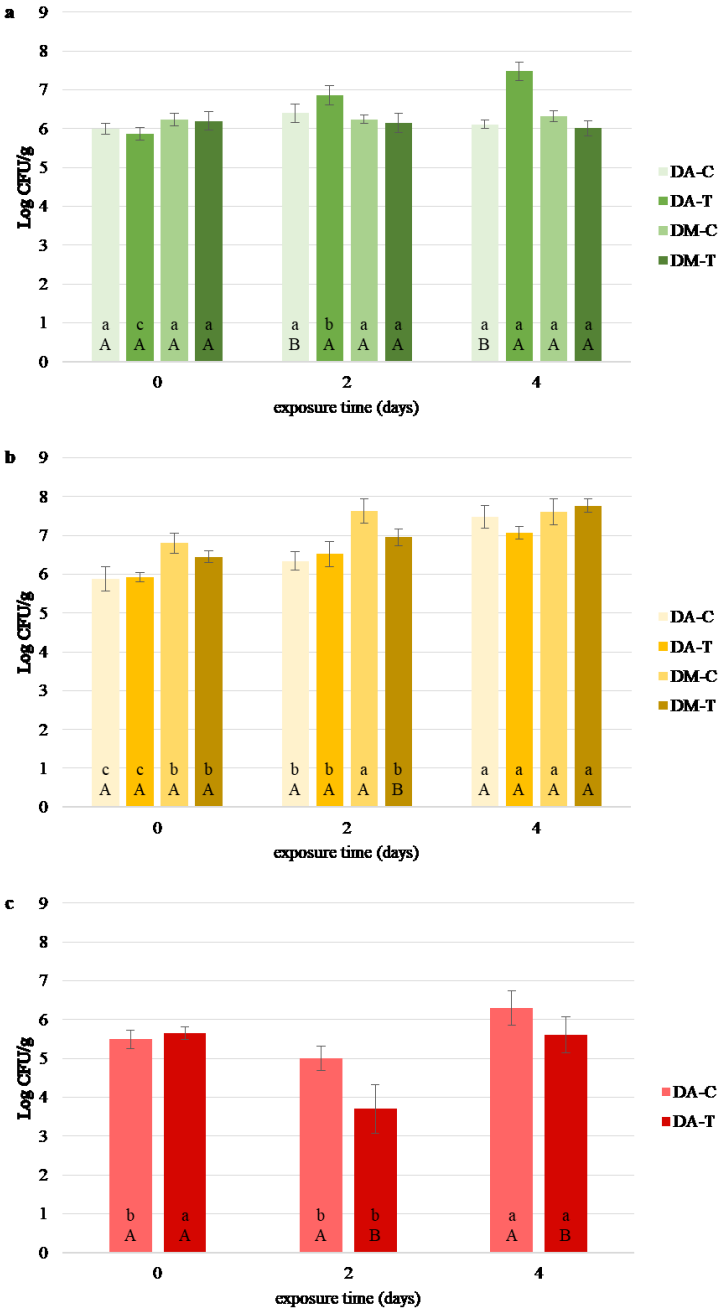

Figure 10 Effect of ionization treatments with DA (4x10^6 NAI/cm^{3}s + 2.20 mg/h O_3) and DM (1x10^7 NAI/cm^{3}s + 0.02 mg/h O_3) on the total bacterial count of lettuce (a), strawberry (b), and mushroom (c). C = control samples; T = treated samples. For the same ionizer, means with different letters within a day (uppercase letter) are significantly different ($p < 0.05$). For the same ionizer and sample, means with different letters (lowercase letter) are significantly different ($p < 0.05$).

3.3.1.3 Conclusions

Considering the results obtained with the ionization treatment on lettuce, strawberry, and mushroom using DA and DM (part 1), the different amounts of NAI and ozone produced resulted to differently affect the appearance of the foods. Lettuce resulted to be an evident color change after the treatment with $4x10^6$ NAI/cm^{3}s + 2.20 mg/h O_3, while for mushrooms was the treatment with $1x10^7$ NAI/cm^{3}s + 0.02 mg/h O_3 that generated a higher browning of the samples. The colorimetric analysis resulted to be valid for supporting the visual observation, giving values of total color change (ΔE^*) in agreement with the ranges proposed by Mokrzycki & Tatol (2011). On the other side, the microbiological assessment indicated that the conditions generated during the treatment were not able to significantly reduce the microbial load of the food samples except for strawberries treated with DA. However, the concentration of ozone generated by this device was considered high to include this type of technology inside the domestic refrigerator, while that generated by DM satisfied the requirements of the OSHA and the Health and Safety Executive about the limits for the exposition to ozone. From these considerations, more investigations were decided to carry out with DM including additional tests on other fruits and vegetables extending also the exposure time to study its predisposition for the treatment of food during refrigerated storage.

3.3.2 Ionization treatment on fruits and vegetables using DM (part 2)

This second part of the preliminary assessment of ionization technology on food involved the use of DM ($1x10^7$ NAI/cm^{3}s + 0.02 mg/h O_3) for the treatment of lettuce, endive, zucchini, tomato, blueberry, and mushroom for 9 days. Additional foods and prolonged time of exposure aimed to verify if the presence of a low concentration of ozone was effectively able to avoid strong oxidation of food components also for longer periods of cold storage, keeping control of the microbial growth for ensuring the safety of the final products. A comparison between the control and the treated foods is reported in Figure 11. The photos were taken at the beginning of the tests (day 0) and the end of the exposure period (day 9). Through a visual observation was possible to perceive that the treatments under low concentration of ozone caused no evident

differences between control and treated samples of lettuce, endive, zucchini, tomato, and blueberry, but affected the treated mushrooms making them browner than the mushroom in the control chamber. However, the treated mushroom did not present the dark spots visible in the control samples, and the browning was more homogeneous on the cap giving a better appearance.

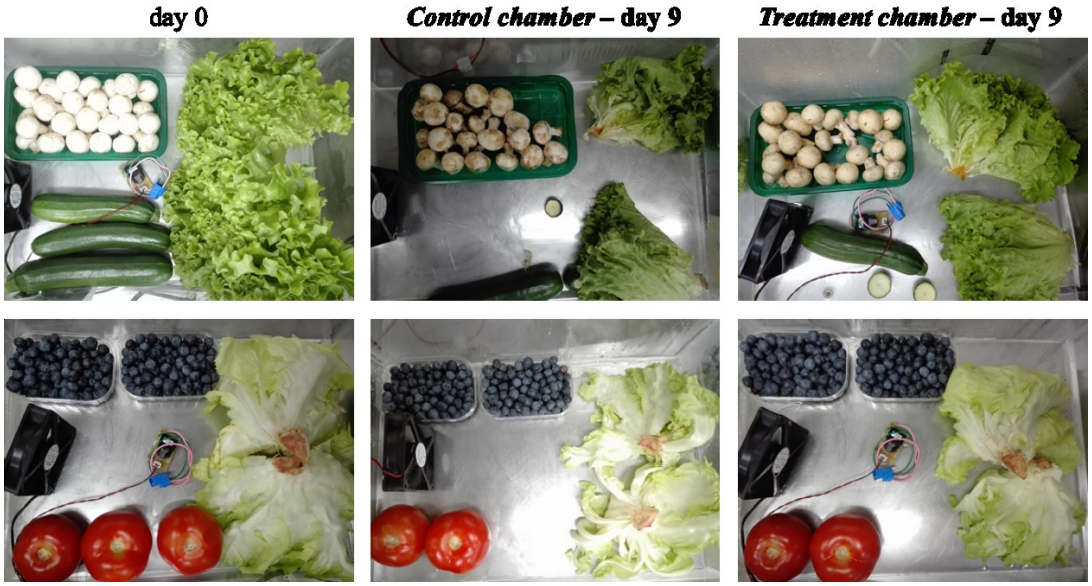

Figure 11 Visual observation monitoring at days 0 and 9 of the ionization treatment on lettuce, endive, zucchini, tomato, blueberry, and mushroom with DM ($1x10^7$ NAI/cm^3s + 0.02 mg/h O_3).

3.3.2.1 Color determinations

The results of color determinations of lettuce, endive, zucchini, tomato, blueberry, and mushroom are reported in Table 16. Generally, for all the food tested, both the control and the treated samples, showed a significant ($p < 0.05$) total color difference (ΔE^*) from the beginning to the end of the ionization treatment. In the cases of lettuce, endive, tomato, and mushrooms the value of ΔE^* was significantly lower ($p < 0.05$) for the treated samples than controls, suggesting a kind of protective effect of NAI in the presence of a low concentration of ozone (Tuffi et al., 2009). On the contrary, for zucchini and blueberry, the ΔE^* values were lower ($p < 0.05$) for the control samples. Nevertheless, all the values were higher than 2, the threshold beyond which also an unexperienced observer can perceive a change in the color (Mokrzycki & Tatol, 2011). However, the results obtained with the color determinations were in contrast with the

visual observation, except for mushrooms, for which the better appearance of the treated samples was evident to the human eye.

The *L*, a*, b*, C**, *H*, and *BI* parameters calculated for the color determination of lettuce, endive, zucchini, tomato, blueberry, and mushroom treated with DM were reported at the end of the chapter in Table 17 and Table 18.

Table 16 Values of ΔE^* (mean ± SD) resulted from the treatment of lettuce, endive, zucchini, tomato, blueberry, and mushroom with DM ($1x10^7$ NAI/cm^3s + 0.02 mg/h O_3).

		day 3	day 7	day 9
Lettuce	control	$2.37 \pm 1.42^{b\,A*}$	$2.77 \pm 0.65^{b\,A}$	$3.20 \pm 0.88^{a\,A}$
	treated	$2.10 \pm 1.35^{a\,A}$	$2.31 \pm 1.95^{a\,A}$	$2.56 \pm 0.63^{a\,B}$
Endive	control	$5.30 \pm 1.45^{a\,A}$	$4.53 \pm 1.27^{a\,A}$	$6.29 \pm 0.99^{a\,A}$
	treated	$1.43 \pm 0.98^{c\,B}$	$3.25 \pm 1.64^{b\,A}$	$5.51 \pm 0.46^{a\,B}$
Zucchini	control	$3.98 \pm 1.13^{c\,A}$	$6.20 \pm 1.63^{a\,A}$	$4.68 \pm 2.49^{b\,A}$
	treated	$2.10 \pm 1.75^{b\,A}$	$5.70 \pm 1.89^{a\,A}$	$6.28 \pm 1.78^{a\,A}$
Tomato	control	$1.70 \pm 3.34^{b\,B}$	$4.71 \pm 1.83^{a\,A}$	$5.61 \pm 2.37^{a\,A}$
	treated	4.02 ± 3.12^{aA}	$2.38 \pm 2.97^{a\,B}$	$2.25 \pm 2.19^{a\,B}$
Blueberry	control	$0.43 \pm 0.58^{c\,B}$	$1.71 \pm 0.74^{b\,B}$	$3.93 \pm 0.71^{a\,B}$
	treated	$2.12 \pm 1.11^{c\,A}$	$4.83 \pm 0.94^{b\,A}$	$6.21 \pm 1.24^{a\,A}$
Mushroom	control	$7.69 \pm 3.58^{b\,A}$	$16.49 \pm 5.50^{a\,A}$	$17.03 \pm 2.69^{a\,A}$
	treated	$7.13 \pm 3.10^{b\,A}$	$12.24 \pm 0.36^{a\,B}$	$13.41 \pm 1.10^{a\,B}$

*For each food, means with different letters within a column (uppercase letter) are significantly different ($p < 0.05$); means with different letters within a raw (lowercase letter) are significantly different ($p < 0.05$).

3.3.2.2 Microbiological analysis

The evolution of total bacterial count during the 9 days of the exposure to ionization treatment under low concentration of ozone on lettuce, mushroom, zucchini, endive, tomato, and blueberry is reported in Figure 12. The arrows indicate the trend of the evolution of microbial load in the treated samples during the 9 days of the exposure to ionization. The microbial count of the blueberries was always below the detection limit of the method (2 Log CFU/g), indicating that this fruit has a low load of natural microflora (Gazula et al., 2019; Quansah et al., 2019), that did not growth during cold storage. Endive, lettuce, and tomatoes showed a significant decrease ($p < 0.05$) in the microbial load after 9 days of treatment with $1x10^7$ NAI/cm^3s + 0.02 mg/h O_3, demonstrating the efficacy of ionization technology to keep control of the microbiological quality of these vegetables (Tuffi et al., 2012). On the contrary, in the case of mushrooms and zucchini, a significant increase ($p < 0.05$) of the total aerobic count occurred

during the 9 days of the treatment. For mushrooms, a similar behavior was found in part 1, confirming the susceptibility of this food to microbial development. Regarding zucchini, the same trend observed in this experiment was identified during other types of treatments aiming to improve their quality and decay (De Bruin et al., 2016), suggesting the difficulty of the treatments in general to affect the natural microflora of this food.

3.3.2.3 Conclusions

The second part of the study confirmed that the treatment with $1x10^7$ NAI/cm^3s + 0.02 mg/h O_3 generated by DM did not affect the overall visual quality of the treated foods after 9 days of the exposition. In addition, in some cases, the microbial load of the treated foods at the end of the treatment was kept stable or was lower than the initial counts, allowing to control their microbiological quality.

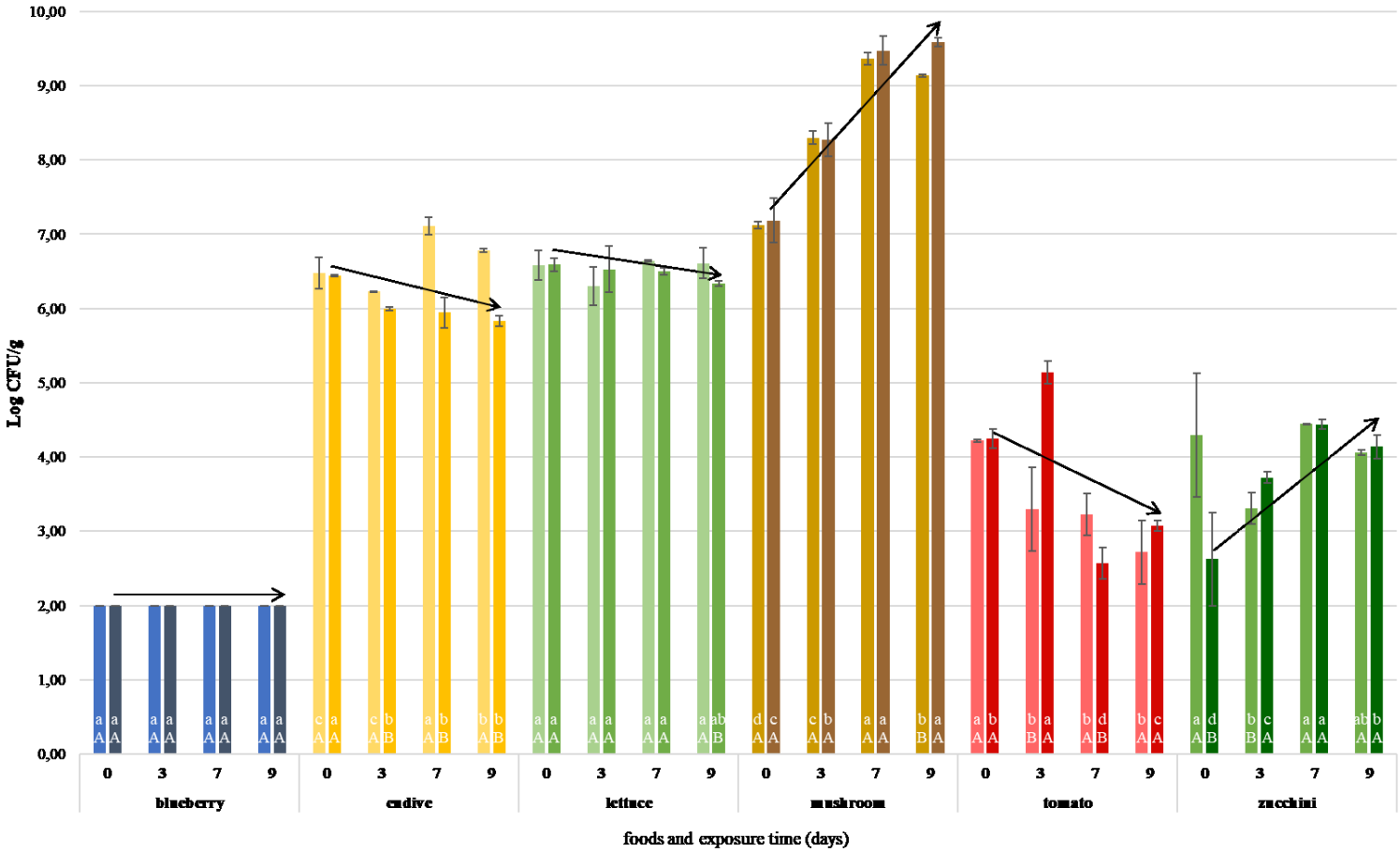

Figure 12 Effect of ionization treatment with DM ($8x10^6$ – $2x10^7$ NAI/cm^3s + 0.02 mg/h of ozone) on the total bacterial count of blueberries (blue), endive (yellow), lettuce (light green), mushroom (brown), tomato (red), and zucchini (dark green). For each day: first lighter bar = control sample; second darker bar = treated sample. Means with different letters within day (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within food category (lowercase letter) are significantly different ($p < 0.05$).

3.4 Criticalities

Despite the good results observed, some critical aspects were found during the trial.

1 The used digital camera and the surrounding conditions (light, food position, …) in which the pictures were taken were not so suitable to obtain pictures comparable between them since standardized settings were not possible to achieve at the moment of the shooting.

2 The method used for the colorimetric analysis was destructive, therefore the samples analyzed at the different days were never the same and a real comparison cannot be done. Although the samples belonged to the same batch in the case of mushroom, strawberry, and blueberries or the same head in the case of lettuce and endive, fruits or leaves are never the same and the results cannot be accurate.

3.5 Conclusions

Considering the results obtained with the first assessment of NAI with different amounts of ozone (2.20 mg/h of DA and 0.02 mg/h of DM) for the treatment of food, DA was not appropriate for this purpose because of the evident oxidation phenomena caused in the tested foods and the higher values of ozone considered non-safe for the application inside a domestic refrigerator. Indeed, $1x10^7$ NAI/cm^3s + 0.02 mg/h O_3 generated by DM did not negatively affect the tested food, exception for mushrooms, for which a slight browning was observed at the end of the treatment, suggesting the possibility to use this combination of NAI and ozone for the maintenance or improving the hygienic status of the refrigerated storage environment.

Table 17 Effect of ionization treatment with DM ($1x10^7$ NAI/cm^3s + 0.02 mg/h O_3) on *L*, a*, b*, C**, *H°,* and *BI* of lettuce, zucchini, and mushroom (mean ± SD).

		lettuce		**zucchini**		**mushroom**	
	day	control	treated	control	treated	control	treated
L*	0	66.60 ± 1.33 $^{a\,A}$	67.04 ± 0.85 $^{b\,A}$	41.10 ± 3.93 $^{a\,A}$	40.66 ± 4.26 $^{a\,A}$	85.66 ± 3.23 $^{a\,A}$	82.20 ± 2.03 $^{a\,B}$
	3	67.25 ± 1.62 $^{a\,A}$	67.58 ± 1.00 $^{b\,A}$	44.76 ± 7.17 $^{a\,A}$	42.70 ± 7.79 $^{c\,B}$	80.43 ± 2.58 $^{b\,A}$	76.60 ± 3.44 $^{b\,B}$
	7	66.73 ± 1.46 $^{a\,B}$	68.30 ± 2.19 $^{a\,A}$	45.56 ± 2.19 $^{a\,A}$	45.43 ± 2.80 $^{a\,A}$	73.64 ± 10.84 $^{c\,A}$	71.79 ± 1.98 $^{c\,B}$
	9	66.43 ± 1.92 $^{a\,A}$	65.97 ± 0.58 $^{a\,A}$	44.77 ± 3.43 $^{a\,A}$	46.88 ± 4.38 $^{a\,A}$	73.06 ± 1.65 $^{c\,A}$	70.68 ± 2.29 $^{d\,B}$
a*	0	-22.29 ± 0.78 $^{a\,A}$	-21.23 ± 0.62 $^{a\,A}$	-6.28 ± 0.48 $^{a\,A}$	-5.33 ± 2.47 $^{a\,A}$	6.29 ± 0.90 $^{c\,A}$	9.76 ± 0.38 $^{c\,A}$
	3	-21.36 ± 0.22 $^{a\,A}$	-22.52 ± 0.64 $^{a\,A}$	-5.29 ± 1.94 $^{a\,A}$	-5.13 ± 1.32 $^{a\,A}$	10.94 ± 0.53 $^{b\,B}$	17.68 ± 0.77 $^{b\,A}$
	7	-21.92 ± 0.52 $^{a\,A}$	-21.51 ± 0.61 $^{a\,A}$	-4.47 ± 1.27 $^{a\,A}$	-3.99 ± 1.21 $^{a\,A}$	17.22 ± 3.10 $^{a\,A}$	21.86 ± 0.44 $^{a\,A}$
	9	-22.40 ± 0.68 $^{a\,A}$	-20.53 ± 0.6 $^{a\,A}$	-5.31 ± 2.23 $^{a\,A}$	-4.77 ± 1.57 $^{a\,A}$	19.09± 0.51 $^{a\,B}$	21.28 ± 0.48 $^{a\,A}$
b*	0	66.14 ± 2.31 $^{a\,A}$	62.04 ± 1.69 $^{b\,B}$	12.14 ± 1.65 $^{a\,A}$	9.67 ± 5.35 $^{a\,A}$	44.88 ± 2.55 $^{c\,B}$	53.27 ± 1.15 $^{c\,A}$
	3	65.14 ± 1.66 $^{a\,A}$	67.24 ± 1.56 $^{a\,A}$	10.79 ± 5.35 $^{a\,A}$	9.19 ± 3.58 $^{a\,A}$	59.68 ± 1.86 $^{b\,A}$	62.33 ± 1.15 $^{b\,A}$
	7	68.38 ± 2.02 $^{a\,A}$	66.16 ± 1.24 $^{a\,A}$	8.23 ± 3.07 $^{a\,A}$	6.86 ± 2.87 $^{a\,A}$	71.82 ± 3.25 $^{a\,A}$	67.91 ± 1.72 $^{a\,A}$
	9	69.80 ± 2.56 $^{a\,A}$	62.66 ± 1.46 $^{b\,B}$	9.40 ± 5.17 $^{a\,A}$	9.00 ± 5.15 $^{a\,A}$	76.40 ± 2.38 $^{a\,A}$	69.79 ± 0.26 $^{a\,A}$
C*	0	23.27 ± 2.44 $^{c\,A}$	21.86 ± 1.80 $^{b\,A}$	13.67 ± 1.74 $^{a\,A}$	11.04 ± 5.86 $^{a\,A}$	15.12 ± 2.61 $^{d\,B}$	18.05 ± 1.18 $^{d\,A}$
	3	22.85 ± 1.63 $^{c\,B}$	23.64 ± 1.68 $^{a\,A}$	12.11 ± 5.61 $^{a\,A}$	10.53 ± 3.76 $^{a\,A}$	20.24 ± 1.77 $^{c\,B}$	21.60 ± 1.27 $^{c\,A}$
	7	23.94 ± 2.03 $^{b\,A}$	23.19 ± 1.32 $^{a\,A}$	9.37 ± 3.33 $^{a\,A}$	7.93 ± 3.12 $^{a\,A}$	24.74 ± 3.39 $^{b\,A}$	23.78 ± 1.73 $^{a\,B}$
	9	24.44 ± 2.58 $^{a\,A}$	21.98 ± 1.56 $^{b\,B}$	10.79 ± 5.53 $^{a\,A}$	10.18 ± 5.25 $^{a\,A}$	26.25 ± 2.35 $^{a\,A}$	24.32 ± 0.37 $^{a\,B}$
H°	0	178.75 ± 0.01 $^{a\,A}$	178.76 ± 0.00 $^{a\,A}$	178.91±0.02 $^{a\,A}$	178.93 ± 0.06 $^{a\,A}$	1.43 ± 0.05 $^{a\,A}$	1.39 ± 0.01 $^{a\,A}$
	3	178.75 ± 0.02 $^{a\,A}$	178.75 ± 0.01 $^{a\,A}$	178.90 ± 0.08 $^{a\,A}$	178.94 ± 0.07 $^{a\,A}$	1.39 ± 0.04 $^{a\,A}$	1.29 ± 0.03 $^{a\,A}$
	7	178.74 ± 0.02 $^{a\,A}$	178.74 ± 0.02 $^{a\,A}$	178.93 ± 0.04 $^{a\,A}$	178.96 ± 0.06 $^{a\,A}$	1.34 ± 0.11 $^{a\,A}$	1.26 ± 0.02 $^{a\,A}$
	9	178.74 ± 0.03 $^{a\,A}$	178.75 ± 0.01 $^{a\,A}$	178.94 ± 0.11 $^{a\,A}$	178.92 ± 0.11 $^{a\,A}$	1.33 ± 0.03 $^{a\,A}$	1.28 ± 0.02 $^{a\,A}$
BI	3	-	-	-	-	0.55 ± 0.35 $^{b\,A}$	0.39 ± 0.24 $^{a\,A}$
	7	-	-	-	-	1.20 ± 0.38 $^{a\,A}$	0.67 ± 0.10 $^{a\,B}$
	9	-	-	-	-	1.39 ± 0.34 $^{a\,A}$	0.74 ± 0.06 $^{a\,B}$

*For each color parameter and food, means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

Table 18 Effect of ionization treatment with DM ($1x10^7$ NAI/cm^3s + 0.02 mg/h O_3) on *L*, a*, b*, C**, and *H* of endive, tomato, and blueberry.

		endive		**tomato**		**blueberry**	
	day	control	treated	control	treated	control	treated
L*	0	77.52 ± 5.50 $^{a A*}$	77.78 ± 6.36 $^{a A}$	40.68 ± 1.88 $^{a A}$	42.26 ± 2.06 $^{a A}$	44.45 ± 6.33 $^{a A}$	46.70 ± 6.08 $^{a A}$
	3	74.74 ± 6.80 $^{a A}$	75.66 ± 6.90 $^{a A}$	42.17 ± 2.37 $^{a A}$	42.06 ± 2.21 $^{a A}$	44.48 ± 6.87 $^{a A}$	44.63 ± 4.95 $^{a A}$
	7	73.06 ± 7.43 $^{a A}$	74.46 ± 6.12 $^{a A}$	42.91 ± 1.29 $^{a A}$	41.40 ± 1.52 $^{a A}$	42.89 ± 5.23 $^{a A}$	42.07 ± 6.61 $^{a A}$
	9	71.99 ± 5.49 $^{a A}$	71.99 ± 6.04 $^{a A}$	43.35 ± 2.87 $^{a A}$	43.48 ± 2.13 $^{a A}$	40.63 ± 6.12 $^{a A}$	40.76 ± 5.59 $^{a A}$
a*	0	-5.98 ± 3.27 $^{a A}$	-5.72 ± 3.40 $^{a A}$	22.87 ± 1.47 $^{a A}$	21.34 ± 1.30 $^{a A}$	1.09 ± 1.01 $^{a A}$	0.38 ± 1.10 $^{a A}$
	3	-6.26 ± 3.30 $^{a A}$	-5.69 ± 3.64 $^{a A}$	22.24 ± 2.04 $^{a A}$	15.63 ± 1.68 $^{b B}$	1.41 ± 1.51 $^{a A}$	1.78 ± 1.14 $^{a A}$
	7	-5.83 ± 3.62 $^{a A}$	-4.68 ± 4.30 $^{a A}$	18.73 ± 2.47 $^{a A}$	19.13 ± 1.43 $^{a A}$	1.55 ± 1.46 $^{a A}$	0.84 ± 1.44 $^{a A}$
	9	-5.95 ± 3.87 $^{a A}$	-4.56 ± 4.19 $^{a A}$	18.69 ± 3.21 $^{a A}$	18.76 ± 2.87 $^{a A}$	1.49 ± 1.32 $^{a A}$	1.46 ± 1.78 $^{a A}$
b*	0	22.08 ± 7.88 $^{a A}$	21.53 ± 8.62 $^{a A}$	19.38 ± 1.12 $^{a A}$	21.19 ± 2.22 $^{a A}$	-3.10 ± 1.73 $^{a A}$	-3.04 ± 1.83 $^{a A}$
	3	23.18 ± 9.43 $^{a A}$	21.50 ± 8.87 $^{a A}$	18.86 ± 2.63 $^{a A}$	17.35 ± 3.08 $^{a A}$	-2.81 ± 1.75 $^{a A}$	-0.93 ± 1.94 $^{a A}$
	7	23.70 ± 7.86 $^{a A}$	21.62 ± 8.88 $^{a A}$	19.12 ± 2.21 $^{a A}$	21.08 ± 1.62 $^{a A}$	-2.57 ± 1.73 $^{a A}$	-2.95 ± 1.94 $^{a A}$
	9	23.98 ± 6.99 $^{a A}$	21.61 ± 8.17 $^{a A}$	19-24 ± 1.89 $^{a A}$	20.98 ± 2.47 $^{a A}$	-2.93 ± 1.81 $^{a A}$	-2.88 ± 1.75 $^{a A}$
C*	0	23.53 ± 8.42 $^{a A}$	22.61 ± 9.24 $^{a A}$	29.98 ± 9.15 $^{a A}$	30.14 ± 2.27 $^{a A}$	3.29 ±1.44 $^{a A}$	3.06 ± 1.14 $^{a A}$
	3	28.04 ± 9.90 $^{a A}$	22.75 ± 9.44 $^{a A}$	29.16 ± 3.22 $^{a A}$	26.95 ± 3.12 $^{a A}$	3.14 ± 1.44 $^{a A}$	3.05 ± 0.76 $^{a A}$
	7	24.03 ± 8.42 $^{a A}$	22.54 ± 9.19 $^{a A}$	26.77 ± 3.12 $^{a B}$	28.46 ± 1.78 $^{a A}$	3.00 ± 1.55 $^{a A}$	3.44 ± 1.37 $^{a A}$
	9	21.14 ± 9.15 $^{a A}$	22.13 ± 8.98 $^{a A}$	25.93 ± 3.99 $^{a A}$	28.88 ± 2.87 $^{a A}$	2.92 ± 1.63 $^{a A}$	2.94 ± 1.13 $^{a A}$
H°	0	178.70 ± 0.06 $^{a A}$	178.69 ± 0.06 $^{a A}$	0.70 ± 0.02 $^{a A}$	0.78 ± 0.04 $^{a A}$	358.77 ± 0.04 $^{a A}$	358.55 ±0.04 $^{a A}$
	3	178.71 ± 0.06 $^{a A}$	178.69 ± 0.08 $^{a A}$	0.70 ± 0.03 $^{a A}$	0.70 ± 0.07 $^{a A}$	358.89 ± 0.03 $^{a A}$	358.71 ± 0.03 $^{a A}$
	7	178.67 ± 0.09 $^{a A}$	178.65 ± 0.05 $^{a A}$	0.80 ± 0.04 $^{a A}$	0.83 ± 0.04 $^{a A}$	358.97 ± 0.04 $^{a A}$	358.97 ± 0.02 $^{a A}$
	9	178.62 ± 0.06 $^{a A}$	178.60 ± 0.06 $^{a A}$	0.80 ± 0.03 $^{a A}$	0.83 ± 0.04 $^{a A}$	359.04 ± 0.03 $^{a A}$	359.17 ±0.03 $^{a A}$

*For each color parameter and food, means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

PART 3

STUDY OF IONIZATION TECHNOLOGY IN A SIMULATED REAL APPLICATION INSIDE A DOMESTIC REFRIGERATOR

The food exposition to ionization technology allowed to select DM as the best device to use in the presence of food at refrigeration temperature since the oxidative damage towards food was very restricted. Therefore, the following study was focused on the assessment of the ionization technology in a real simulation of application in the domestic refrigerator. The ionizer was placed inside the drawer for the storage of fruits and vegetables. Since the study on food had already given promising results, the evaluation was focused firstly on the assessment of the technology to affect the microbial population, at refrigeration temperature, on different surfaces made of materials generally used for making the internal cavity of the refrigerator. Therefore, the evaluation was done on selected foods, to investigate the effect of ionization on food in a real condition of storage. The final aim was to understand if the use of NAI with a low concentration of ozone can improve the hygienic status of the surrounding environment without causing adverse effects on food.

4 ANTIMICROBIAL EFFECT OF IONIZATION TECHNOLOGY ON DIFFERENT SURFACES

4.1 Introduction and aim of the study

The domestic refrigerator is one of the most important ways to maintain food freshness and to prevent food spoilage. However, in the food, a natural microflora is present, and therefore, during the conservation, cross-contamination between food and surfaces can occur (Ye et al., 2019). Different values of the total viable count were found in domestic refrigerators, ranging from 1 to 7 Log CFU/cm; the microbial species identified are many and belonged to *Enterobacteriaceae* and *Bacillaceae* families, *Pseudomonas* spp., *Aeromonas* spp., *Salmonella* spp., *Penicillium* spp. *Aspergillus* spp., *Saccharomyces* spp., and *Candida* spp. (Carpentier et al., 2012; Catellani et al., 2014; Kennedy et al., 2005; Vegara et al., 2014; Ye et al., 2019). The levels of contamination are influenced by several factors, like the initial contamination of foods, the presence of packaging, and the efficiency and frequency of refrigerator maintenance and cleaning (Jackson et al., 2007). The correct cleaning procedures of the domestic refrigerators performed by the consumers represent, therefore, one of the practices to maintain the microbiological quality at safe levels, but unfortunately, most of them neglect their role regarding domestic refrigerator management (Vegara et al., 2014). Trying to supply this lack, some strategies were approached by the food industry, like surfaces containing silver, photocatalytic filters, or ionization modules demonstrating to obtain

good results in terms of implementation of the hygienic status of the refrigerators (Ilg et al., 2011; Kampmann et al., 2008, 2009; Yu et al., 2008).

The study aimed to investigate the antimicrobial effect of ionization technology at refrigeration temperature towards *B. subtilis*, *E. coli*, *L. monocytogenes*, *Ps. fluorescens*, *P. roqueforti*, and *S. cerevisiae* on glass, polypropylene, and polystyrene, the main materials used for the realization of inner surfaces of a domestic refrigerator.

4.2 Materials and methods

4.2.1 Microbial cultures

The microorganisms used for the assessment of the antimicrobial effect of ionization technology on surfaces are described in section 2.2.1.

4.2.2 Culture media

The culture media for the microbial cultures were described in section 2.2.2.

4.2.3 Preparation of surfaces

The antimicrobial effect was investigated on three different materials: glass, polypropylene, and polystyrene. The coupons (18 mm x 18 mm) were immersed in 70% ethanol (Carlo Erba, Milan, Italy) and 5% hydrogen peroxide (Sigma-Aldrich, Milan, Italy) for 1 h for sterilization, rinsed with sterile deionized water and left in the lamination chamber until complete drying.

4.2.4 Preparation of the specimens

From the overnight suspensions, dilutions of each strain were done in the relative broths to obtain a final concentration of 10^5-10^6 CFU/mL (stock suspension). 100 µL of the stock suspension was distributed on the surfaces with 10 drops of 10 µL to cover the surface as homogeneously as possible. The surfaces were left in the lamination chamber for 30 min and therefore used for the ionization test.

4.2.5 Operative conditions

To assess the selected ionizer in the final use case, a refrigerator (llt9va52u, Electrolux) was used. The investigations were conducted inside the two vegetable drawers of the

refrigerator. Drawer 1 (18 cm x 31 cm x 32 cm; H x L x W) had a volume of 0.018 m^3, drawer 2 (18 cm x 43.5 cm x 32 cm; H x L x W) had a volume of 0.025 m^3. The ionizer was fixed in the upper part of drawer 2 (Figure 13), while the other drawer served as a control. As a preliminary step before the ionization tests, two conditions were evaluated:

1 Assessment of the trend of the temperature and relative humidity inside the two drawers was done for 24 h using a digital humidity sensor (SHT7x, Sensirion). The data were collected every 60 s with a data logger (evaluation kit EK-H4, Sensirion).

2 Monitoring of movement of NAI and ozone from drawer 2 to drawer 1 was done for 4 h with ion counter (Air Ion Counter Model AIC2, AlphaLab Inc., Salt Lake City, UT, USA) and ozone analyzer (Model UV-100 Serial, Eco Sensors, Santa Fe, NM, USA). In addition, for further verification of the possible movement of the active species generated, some plates inoculated with *B. subtilis* were placed without lids inside both drawers. Plates with lid were also placed in drawer 1. The ionizer was switched on for 4 h and then the plates were incubated at 30 °C for 24 h.

Temperature and relative humidity parameters were always monitored during the tests with the same sensor (SHT7x, Sensirion). A fan was not included because the fridge already had its ventilation. Before the exposure to the ionization treatment, the drawers were cleaned with ethanol 70% (v:v).

Figure 13 Refrigerator model (a), position of the ionizer in drawer 2 (b), and probes position for the monitoring of temperature and relative humidity (c).

4.2.6 Exposition to ionization treatment

The specimens prepared as reported in section 4.2.4 were positioned inside Petri dishes to simplify handling and then the dishes without lids were placed inside the drawers and exposed to the ionization treatment for 24 h, 4 days, and 7 days. After the treatment, the specimens were transferred in a tube containing 3 mL of MRD and 8 beads Ø 5 mm and vortexed for 1 min. Decimal dilutions of the resulting suspension were done, plated on the relative agar medium according to the drop plate method (Herigstad et al., 2001), and incubated at specific conditions (*E. coli* and *L. monocytogenes* at 37 °C for 24 h, *B. subtilis*, *Ps. fluorescens* and *S. cerevisiae* at 30 °C for 24 h, and *P. roqueforti* at 22 °C for 72 h). Finally, the count of viable cells was done.

4.2.7 Observation of bacteria cells using scanning electron microscopy (SEM)

The effect of oxidative species generated during ionization treatment on cells of *E. coli* was evaluated using SEM (FEI Quanta 400). Polystyrene specimens after 7 days of treatment and relative controls were fixed in 4% glutaraldehyde solution in Phosphate Buffered Saline (PBS, Sigma-Aldrich, Milan, Itlay) for 1 h. Then, samples were immersed for 10 min in a series of ethanol solutions (25%, 50%, 75%, 90%, and 100%) for dehydration and dried at 60 °C for 10 min (Weerarathne et al., 2021). Specimens were stored at refrigeration conditions until the visualization with SEM at 5000 – 40000 x magnifications with a Single Shot Detector (SSD) in low vacuum.

4.2.8 Statistical analysis

Each trial was carried out in at least two biological replicates and for each of these, two technical repetitions were done. The means obtained from replicate tests were subjected to one-way analysis of variance ($p < 0.05$), preceded by the Levene test to verify the homogeneity of variance between means using Statistics 8.0 (Statsoft software, Tulsa, Oklahoma, USA). Differences between the means were assessed using Tukey's HSD post-hoc test.

4.3 Results and discussion

4.3.1 Operative conditions

The refrigerator used for this part of the activities was an American model widely used in the company for carrying out tests on food preservation. Since the two drawers had different volumes, before starting the tests, an assessment of the trend of relative humidity and temperature was done inside the two drawers in no-load condition to verify that the environmental conditions during the ionization treatment on surfaces would be the same for control and treated samples. The results are reported in Figure 14.

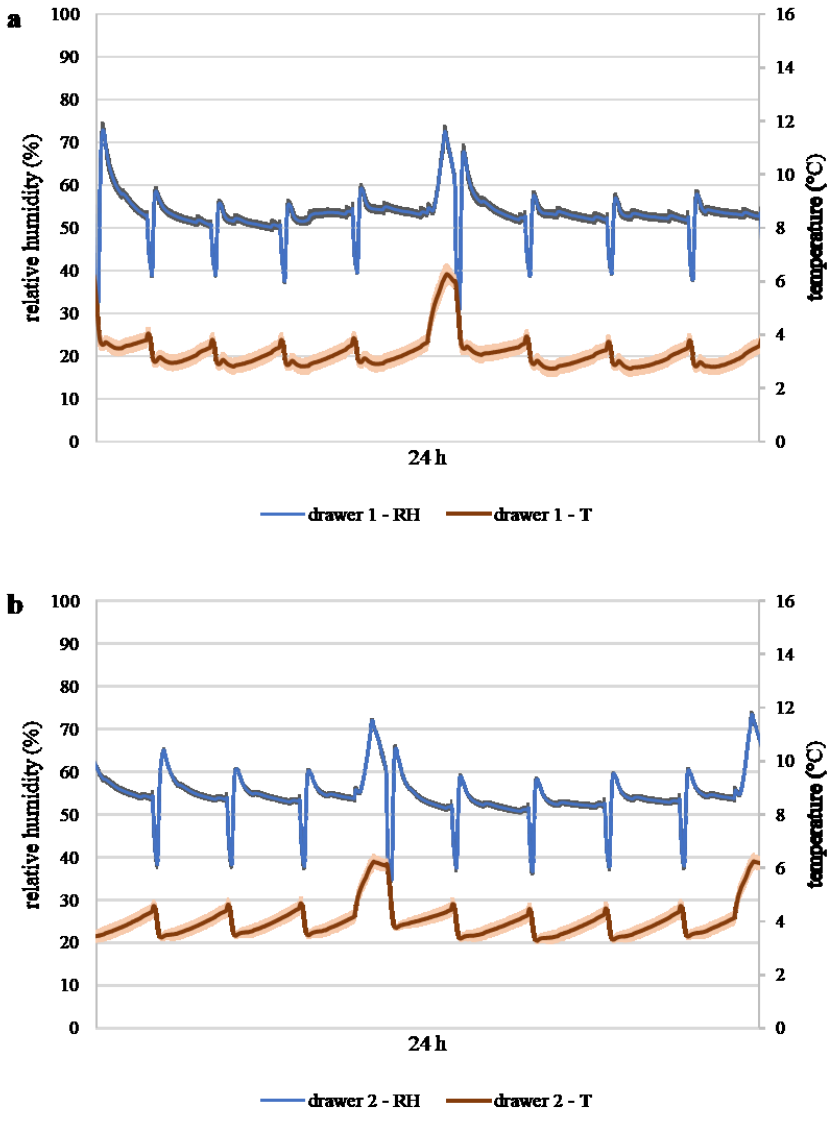

Figure 14 Trend of relative humidity and temperature inside drawer 1 (a) and drawer 2 (b).

The average values of relative humidity were 56 ± 7% RH, with a maximum of 72% and a minimum of 31%, in drawer 1, and 55 ± 8% RH, with a maximum of 72% and a minimum of 35%, in drawer 2. The average values of temperature were 3.4 ± 0.7 °C, with a maximum of 6.2 °C and a minimum of 2.9 °C, in drawer 1, and 4.0 ± 0.7 °C, with a maximum of 6.2 °C and a minimum of 3.4 °C, in drawer 2. Considering these data, as can be seen also from Figure 14, the performances of the two drawers did not present significant differences ($p > 0.05$) neither for relative humidity nor for temperature, therefore the environmental conditions were the same for control and treated samples. Another important evaluation before the ionization tests was to verify if the active species generated by the ionizer moved from drawer 2 to drawer 1 and thus if the last one could be effectively used as a control drawer. The results obtained with the ion analyzer showed that the concentration of NAI in drawer 2 was in the range of 10^6-10^7 NAI/cm^{3}s, confirming the results obtained with the characterization of the DM (section 2.3.1), while the concentration of NAI in the drawer 1 was never exceeding 10^3 NAI/cm^{3}s, indicating that there was no movement of negative air ions from drawer 2 to drawer 1. The monitoring of ozone indicated that the concentration of ozone was always below 0.02 mg/m^3 (the detection limit of the instrument) which was a lower value compared to previous tests performed to characterize the device that was conducted in an empty air-tight chamber (section 2.3.1). The reason for this difference can be related to the not complete tightness of the vegetable drawers of the refrigerator.
Therefore not having the possibility to appreciate any possible difference between the drawers regarding the concentration of ozone, a further verification was performed: some plates inoculated with *B. subtilis* were positioned inside both drawers and the ionizer was switched on for 4 h. This microorganism was selected among all those considered because was the bacteria for which the highest values of Log reduction in the model systems assessments were obtained.

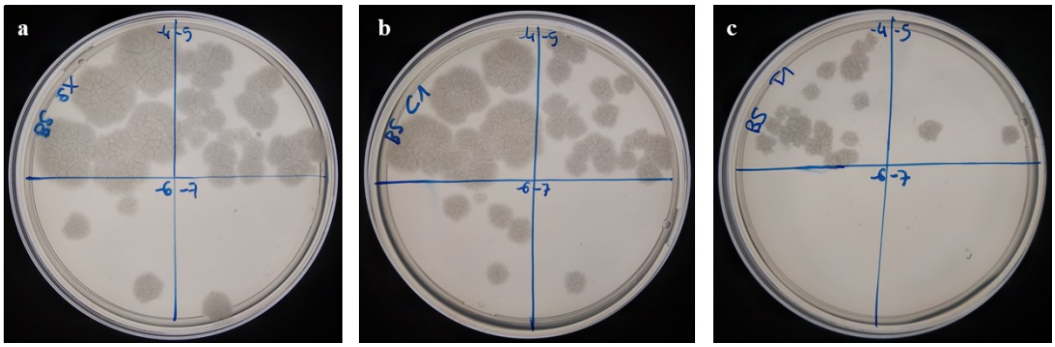

Figure 15 Growth of *B. subtilis* after the exposition to ionization for 4 h inside the refrigerator. a) plate with lid in drawer 1 (negative control), b) plate without lid in drawer 1 (positive control), c) plate without lid in drawer 2 (where the ionizer was fixed; treated sample).

The bacteria test showed that the growth of the cells in the plates of drawer 2, where the ionizer was placed, was 6.60 ± 0.12 Log CFU/mL and was significantly lower ($p < 0.05$) than the negative and positive controls (plates in drawer 1). Moreover, no difference ($p > 0.05$) between closed and opened plates of drawer 1 was observed (7.72 ± 0.23 Log CFU/mL and 7.73 ± 0.16 Log CFU/mL, respectively) (Figure 15). In conclusion, drawer 1 can be used as the control drawer because the environmental conditions were the same as drawer 2 and there was no movement of the NAI and ozone generated by the ionizer from drawer 2 to drawer 1.

4.3.2 Exposition to ionization treatment

B. subtilis, *E. coli*, *L. monocytogenes*, *Ps. fluorescens*, *P. roqueforti*, and *S. cerevisiae* were distributed on the surfaces of three different materials and exposed to ionization treatment under low concentration of ozone ($1x10^7$ NAI/cm^3s + 0.02 mg/h O_3) at refrigeration temperature. The temperature and relative humidity parameters monitored during the tests resulted in 5.2 ± 0.8 °C and 76 ± 12% RH. The materials selected were glass, to simulate the glass shelves, polypropylene, and polystyrene, to simulate refrigerator inner liners and components. Figure 16, Figure 17, and Figure 18 show the bactericidal effect of ionization on the different microorganisms and surfaces. The adhesion of the bacteria to a surface is strictly related to the surface material properties like charge, hydrophobicity, roughness, and topographical configuration (Achinas et al., 2019). Glass has a smooth and hydrophilic surface while plastic materials are more rough and hydrophobic therefore the attachment is favored in these (Berne et al., 2018). The concentrations of *B. subtilis* on day 0 (inocula controls) were 3.89 ± 0.21

Log CFU/cm^2 on glass, 4.24 ± 0.28 Log CFU/cm^2 on polypropylene, and 4.13 ± 0.22 Log CFU/cm^2 on polystyrene. It resulted in better survival on the plastic materials. Indeed, after 1 day the concentrations on the control coupons were 3.21 ± 0.10 Log CFU/cm^2 on polypropylene and 3.35 ± 0.04 Log CFU/cm^2 on polystyrene but 1.57 ± 0.19 Log CFU/cm^2 on glass. After 7 days, the microbial count was lower than days 1 and 4 in all the materials. Focusing on the treated samples of *B. subtilis*, any effect of the ionization was not observed in the glass treated samples during the 7 days. In the treated polypropylene the microbial survival decreased significantly ($p < 0.05$) over time.

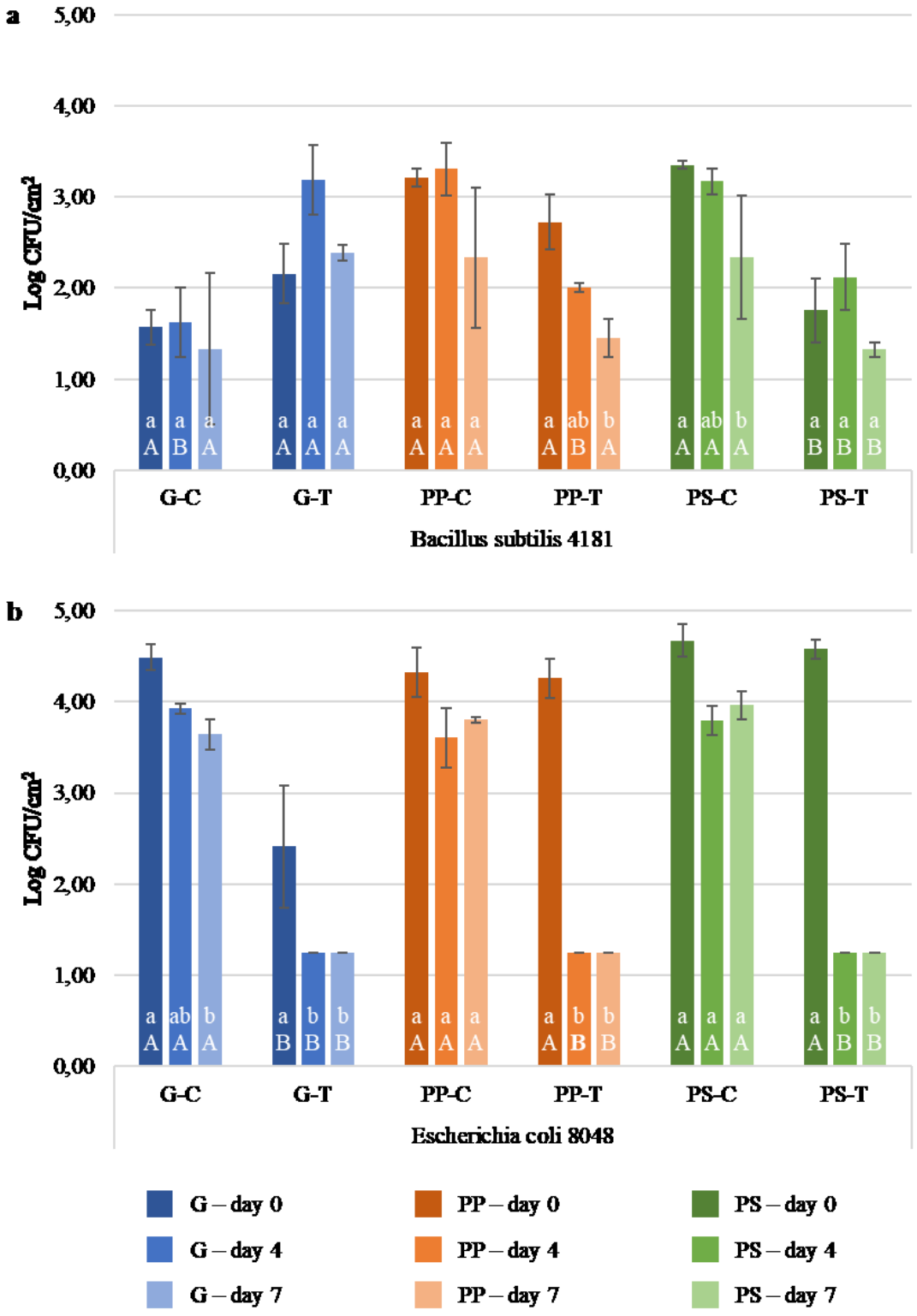

Figure 16 Antimicrobial effect of DM ($1x10^7$ NAI/cm^3s + 0.02 mg/h O_3) on *B. subtilis* (a) and *E. coli* (b) on glass (G), polypropylene (PP), and polystyrene (PS). C = control sample, T = treated sample. Means with different letters are significantly different ($p < 0.05$) for each material, uppercase letters compare control and treated samples on the same days; lowercase letters compare the same samples on different days.

Considering polystyrene, the treatment affected ($p < 0.05$) the viability already after 1 day compared to the control sample on the same day, and the trend was maintained until the end of the exposure time. After 7 days, approximately 2 Log reductions were achieved on the plastic materials. A previous study concerning the ionization treatment inside domestic refrigerators on *B. subtilis* inoculated on glass and plastics demonstrated the efficacy of this technology to reduce its viability after 3 days (Kampmann et al., 2009). Another Gram-positive bacteria studied was the pathogenic *L. monocytogenes*. Inocula controls were 4.87 ± 0.21 Log CFU/cm^2 for all the materials. As for *B. subtilis*, after 1 day the survival was better on plastics materials (4.38 ± 0.16 Log CFU/cm^2 on polypropylene and 4.45 ± 0.16 Log CFU/cm^2 on polystyrene) than on glass (3.61 ± 0.16 Log CFU/cm^2). The bacterial concentration on plastic control samples remained unchanged during the 7 days ($p > 0.05$). The glass control samples showed to have no difference by statistical analysis, however, the bacterial enumeration was variable during the duration of the test. The ionization treatment resulted to affect the viability of *L. monocytogenes* on all the materials. On the glass, the count after 1 and 7 days was lower than the detection limit of the method (1.25 Log CFU/cm^2), while after 4 days the results were more undefined, probably because the smooth glass surface did not allow adhesion of the cells and the stressful condition due to the oxidative species led to the microbial death. *L. monocytogenes* on the polypropylene treated samples showed a significant reduction ($p < 0.05$) already after 1 day of exposure to NAI and on polystyrene treated samples after 4 days compared to control samples. The reduction achieved for *L. monocytogenes* was about 2 Log. These results were interesting considering that *Listeria* is a psychrotrophic microorganism and therefore able to survive at refrigeration temperature (-0.5 – 9.3 °C) (Ferreira et al., 2014; Walker et al., 1990). Also *Ps. fluorescens* is psychrotrophic bacteria. However, starting from an inocula concentration of 4.56 ± 0.09 Log CFU/cm^2 in all the materials, it maintained the same viability in all the control and treated samples on the first day, but after 4 days the viability was significantly ($p < 0.05$) affected in all the samples, both controls and treated. Moreover, the entity of ionization treatment was very significant after 4 days for all the treated materials, maintaining the trend until the end of the exposure period. After 7 days the entity of treatment was 3.5 Log reduction on all materials.

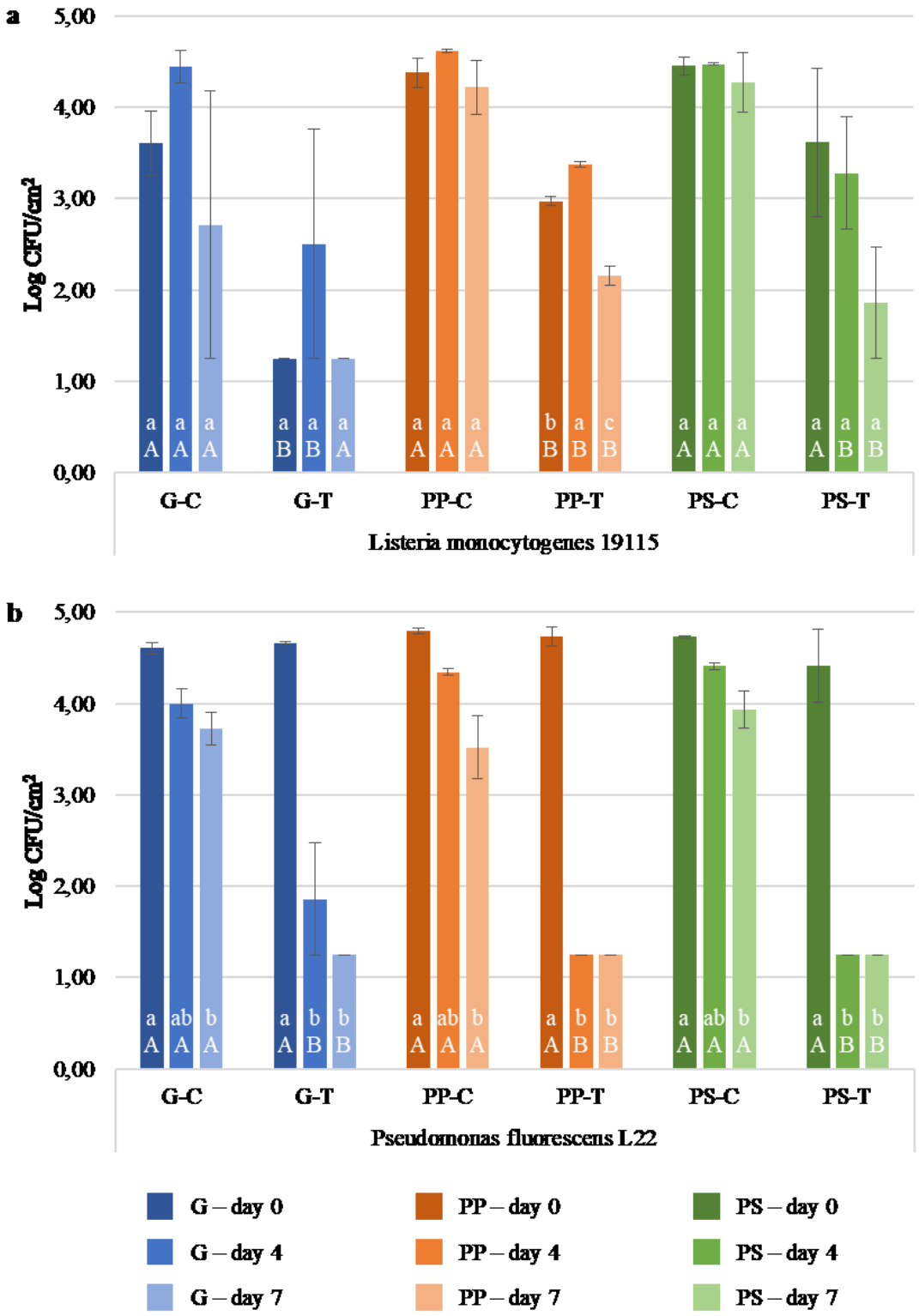

Figure 17 Antimicrobial effect of DM ($1x10^7$ NAI/cm^3s + 0.02 mg/h O_3) on *L. monocytogenes* (a) and *Ps. fluorescens* (b) on glass (G), polypropylene (PP), and polystyrene (PS). C = control sample, T = treated sample. Means with different letters are significantly different ($p < 0.05$) for each material, uppercase letters compare control and treated samples on the same days; lowercase letters compare the same samples on different days.

The results of *Ps. fluorescens* were particularly interesting since the ionization treatment on the model systems tests with DM did not allow any reduction in the viability (see section 2.3.5). However, the different conditions in which the bacteria was dropped, agar medium versus abiotic surfaces, could influence its growing capability and therefore its survival. The findings of the surface tests represent a very important outcome considering that *Pseudomonas* is one of the microorganisms that colonized the internal surfaces of the domestic refrigerator with the *Bacillus* genus and *Enterobacteriaceae* family (Ye et al., 2019). In this study, as representative species of this family, *E. coli* was considered. The concentration of the inocula samples was 4.75 ± 0.03 Log CFU/cm^2 and it was maintained in all the control and treated samples after 1 day except for the treated glass. The trend of the treatment is very similar to that of *Ps. fluorescens*, showing a very important effect of the technology after 4 and 7 days with the count lower than the detection limit of the method in all the treated materials. The bactericidal effect of NAI and low concentration of ozone towards *E. coli* deposited on a membrane was previously found by Park et al. (2016), who showed that the exposure to ions and ozone led to oxidative stress with the increase of intracellular Reactive Oxygen Species (ROS) that oxidized DNA deoxyguanosine leading to cell death. The last two microorganisms tested were two fungi: *S. cerevisiae*, a yeast, and *P. roqueforti*, a mold. While for *P. roqueforti* the ionization treatment cannot affect the viability independently from the type of material or the exposure period, *S. cerevisiae* showed sensitivity towards the active species generated during the treatment by DM. The count on the glass, polypropylene, and polystyrene control samples after 1 day reflected the inocula counts (4.75 ± 0.40 Log CFU/cm^2) and remained stable until day 7. On the contrary, the treatment affected the viability of the yeast, increasing the entity during the whole exposure period. In detail, the ionization treatment generated a significant ($p < 0.05$) viability reduction after day 1 for glass, after 7 days for polypropylene, and after 4 days for polystyrene. At the end of the exposure time, almost 2 Log reductions were achieved for *S. cerevisiae* on all the materials. The ionization treatment under low concentrations of ozone (1x10^7 NAI/cm^{3}s + 0.02 mg/h O_3) at refrigeration temperature was able to affect the vitality of most of the tested microorganisms on the materials typically used for making a domestic refrigerator.

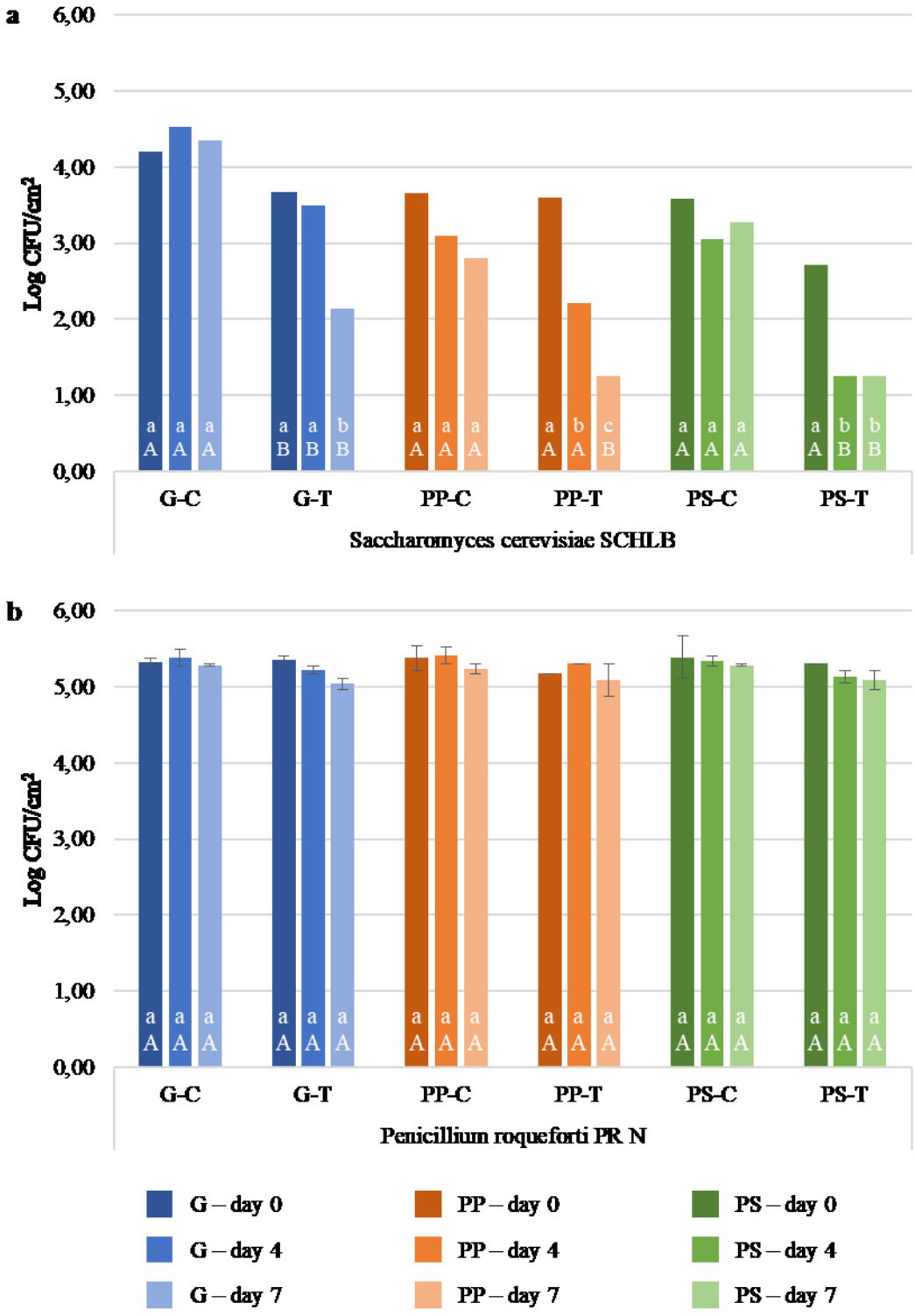

Figure 18 Antimicrobial effect of DM ($1x10^7$ NAI/cm^3s + 0.02 mg/h O_3) on *S. cerevisiae* (a) and *P. roqueforti* (b) on glass (G), polypropylene (PP), and polystyrene (PS). C = control sample, T = treated sample. Means with different letters are significantly different ($p < 0.05$) for each material, uppercase letters compare control and treated samples on the same days; lowercase letters compare the same samples on different days

Among the microorganisms, the pathogen *L. monocytogenes* was affected by the ionization technology that achieved 2 Log reduction for the different tested surfaces.
The results obtained in this part acquired an important meaning considering the possibility to use ionization as a technology able to keep controlled or improve the hygienic status of the refrigerators without affecting the food appearance.

4.3.3 Observation of bacteria cells using scanning electron microscopy (SEM)

The effect on microbial cells of oxidative species generated during the ionization treatment was evaluated on *E. coli* using SEM in a low vacuum modality. It was selected among the species tested since it was the microorganism for which the highest effect of treatment was observed on model systems tests (sections 2.3.3 and 2.3.5). The visualization was done after 7 days of ionization treatment using DM on polystyrene samples at different magnifications to appreciate as better as possible the effect of the treatment (Figure 19). The difference between the control and treated samples was evident. Control cells were whole and well separated from each other, while the cells exposed to the treatment were shrunken and ghost and the cytoplasmatic material appeared to be flowing outside the cells (Tyagi & Malik, 2010). This behavior can be observed in Figure 19b, where the sample appeared as an indistinct mass of cell and intracellular content, showing that the exposure to NAI and ozone at low concentration broke up the cells end eroded or split the cell surface (Y. S. Kim et al., 2011). A similar result on *E. coli* was reported by Tyagi & Malik (2012) after the exposition to NAI and lemongrass oil vapors. They observed that the control cells were intact, rod shape, separated from each other, turgid, and whole with smooth surface, while the treated cells were deformed, ghost, shrunk with the presence of leaked cellular content. The analysis with SEM allowed to have further confirmation of the antimicrobial effect of ionization technology showing how the oxidative species generated during the treatment induced a physiological change in the cell wall structure. Ions generated during a treatment chemically react with the microbial cell through free-radical mechanisms leading to the destruction of the cell and, therefore, to its death (Digel et al., 2005). Moreover, the adopted method for the SEM analysis of the microbial cells was simple to perform and the sample could be directly observed with the microscope.

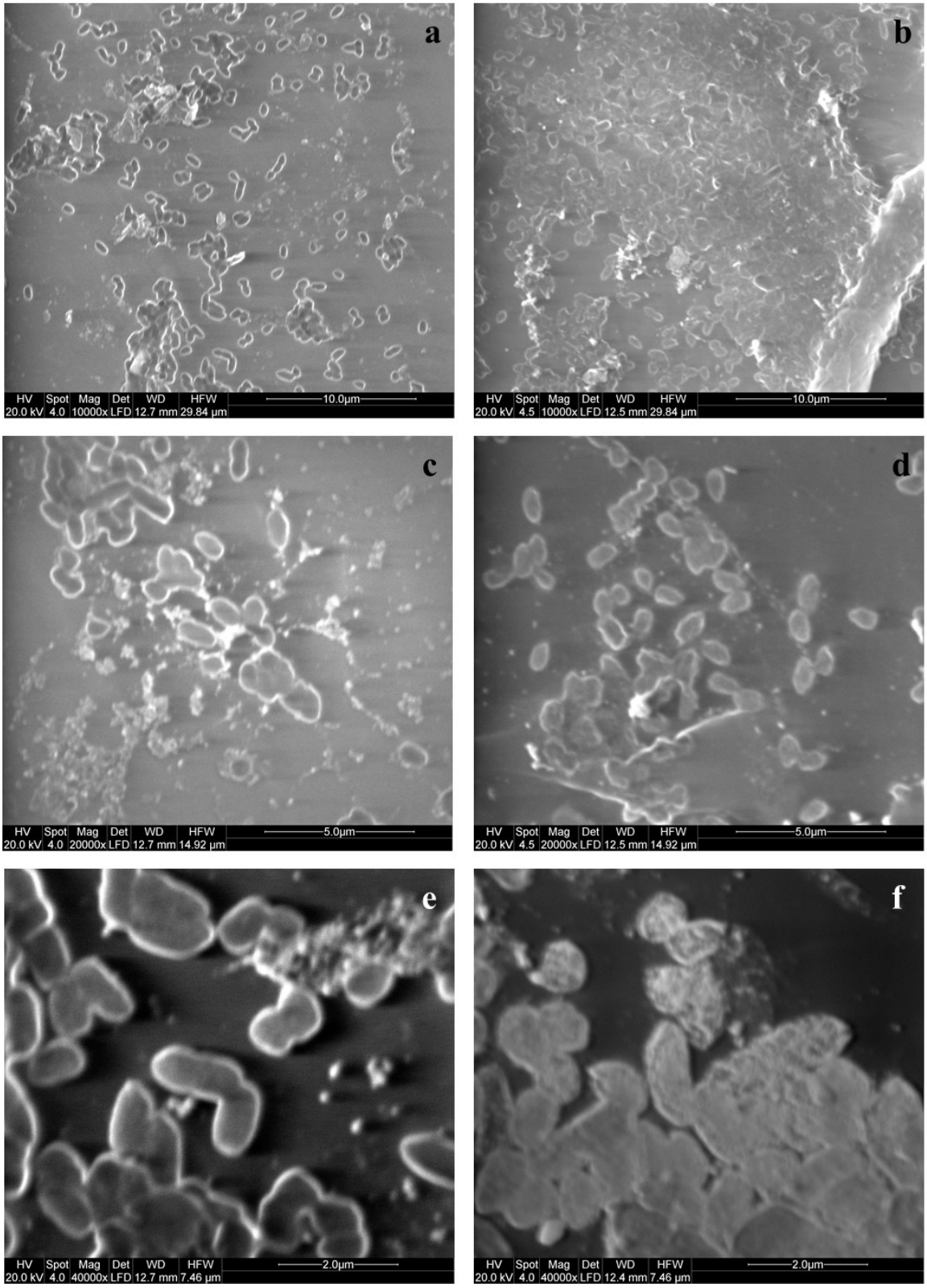

Figure 19 SEM images of *E. coli* after 7 days of ionization treatment with DM ($1x10^7$ NAI/cm^3s + 0.02 mg/h O_3) on polystyrene. (a) control and (b) treated samples at 10000 x magnification; (c) control and (d) traeted samples at 20000 x magnification; (e) control and (f) treated samples at 40000 x magnification.

4.4 Conclusions

The ionization treatment at refrigeration temperature using DM ($1x10^7$ NAI/cm^3s + 0.02 mg/h O_3) resulted to be valid to achieve, within specific application conditions, an antimicrobial effect on B. *subtilis*, *E. coli*, *L. monocytogenes*, *Ps. fluorescens*, and *S. cerevisiae*, but not on *P. roqueforti*. The entity of the treatment was related to the bacteria species, the time of the exposure and the material used. The consequences of the presence of the oxidizing agents on microbial cells were studied on *E. coli* through SEM analysis confirming the potentiality of this technology to provide an antimicrobial effect. Concluding, ionization technology could be used to maintain or improve the microbiological quality of the inner surfaces of a domestic refrigerator.

5 IONIZATION EFFECT ON LETTUCE, MUSHROOM, AND STRAWBERRY AT REFRIGERATION TEMPERATURE

5.1 Introduction and aim of the study

The use of refrigerated storage of fresh food like fruits or vegetables after harvests is one of the most common methods used for their preservation. In recent years, several studies were carried out using novel non-thermal technology to improve the quality of the foods and to maintain as long as possible the fresh-like characteristics (Zhang et al., 2019). In particular, in the domestic environment, the consumers can be supported by applying inside the refrigerator devices able to maintain the hygienic status of the air and surfaces and therefore to increase the shelf-life of the stored food (Kampmann et al., 2008, 2009).

In this study, the effect of ionization technology at refrigeration temperature on lettuce, mushroom, and strawberries was investigated. These foods were selected because they were reported in the company's internal protocol for the evaluation of the shelf-life of fresh products. The aim of the study was twofold:

1 obtain most information as possible by the color imaging system and the following data processing using specific software;
2 develop a protocol for the image analysis of lettuce.

5.2 Materials and methods

5.2.1 Materials

Lettuce (*Lactuca sativa* var. *crispa*), strawberry (*Fragaria x ananassa*), and mushroom (*Agaricus bisporus*) were purchased from a local market (Porcia, PN, Italy) and were stored at 4.7 ± 0.3 °C at 74 ± 13% RH for 2 h under plastic bags until the treatment to prevent any modification of the food before the test. Before the treatment, soil residuals were taken off from mushrooms, leaves with discoloration, spots, or defects from lettuce and endive, and strawberries with defects were removed from the fruit box. In the end, the most possible homogeneous food samples were used for testing.

5.2.2 Operative conditions

The operative conditions for the ionization tests on foods were the same as the tests on abiotic surfaces and were described in section 4.2.5.

5.2.3 Food exposition to ionization treatment

Lettuce, mushrooms, and strawberries were positioned inside the drawers and exposed to the ionization treatment for 14 days. On days 0, 7, and 14 foods were taken and image analysis, weight loss, pH analysis, and microbiological analysis were carried out.

5.2.4 Image analysis

A color imaging system, or vision machine, was used for the documentation of the photos (Figure 20). It consisted of:

1 color digital camera (Nikon D3300, settings: manual mode, shutter speed 1/125 s, F 5.6, ISO 100) fixed perpendicular to the sample;
2 computer with image acquisition software (digiCamControl, ver. 2.0.0.0 for Windows, Copyright© 2015) and MATLAB® software (The Math Works, Inc., USA) for segmentation and analysis of pixel colors;
3 lighting system, using two parallel lamp housings with two compact fluorescent tubes in each housing (Kaiser R1 System, model RB 5055 HF, 4×55 w) with a color temperature of 5400 K (daylight-type, color rendition index (CRI) 90 – 100), and provided with light diffusers (Kaiser Diffusion Screens 5593).

Both lamp housings (60 cm long). The lamps were situated 35 cm above the sample, and the angle between the camera lens and the lighting source axis was approximately 45°;

4 photo chamber to avoid external light reflections during the image acquisition.

For taking photos, the lamps were turned on 30 min before using the vision machine to give them the time to warm up. A matte black soft pad is used to place the individual food pieces on to facilitate the automatic recognition of the true-color image of the food and calculation of changes in color and shape.

Figure 20 Color imaging system: (a) color digital camera, (b) lighting system, (c) matte black soft pad, (d) photo chamber, (e) computer directly connected to the digital camera.

The protocol for the use of the vision system elaborated by the company reported the image analysis method for mushroom and strawberry, but the method for the image analysis of lettuce was not already defined. Therefore, before starting the actual ionization tests on the foods, the development of a protocol was done for this food.

5.2.4.1 Image analysis of strawberries and mushrooms

The protocol of the strawberry image analysis was established to use five fruits per test positioned with the final part of the fruit upwards. The stalks were cut allowing the fruits to stand steadily (Figure 21a).

The protocol for the image analysis of mushrooms considered the study of two sides: the side of the cap (A) and the side of the stipe (B). For each side, three mushrooms were analyzed (1, 2, 3) for a total of 6 mushrooms per test. For the image analysis of

side A, the stipe was cut until just below the cap making sure that the mushrooms can stand steadily (Figure 21b).

The image analysis processing evaluated singularly each mushroom or strawberry and the final values of the color parameters were calculated taking the average of all the samples.

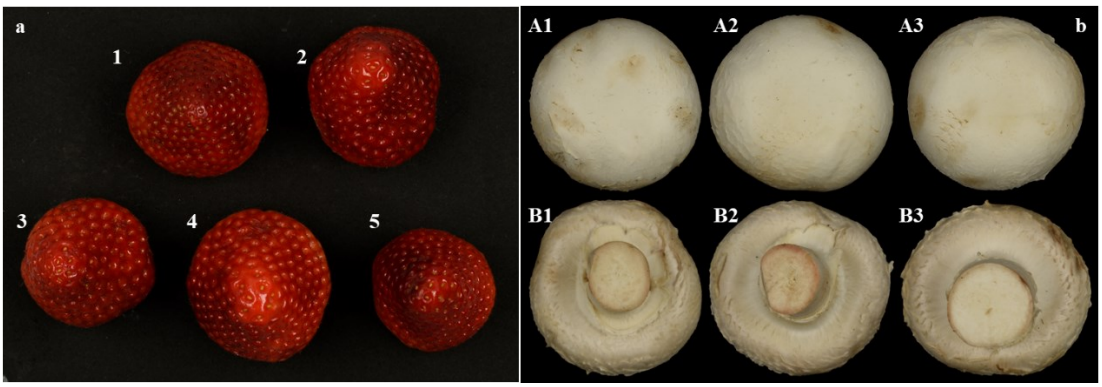

Figure 21 Example of image analysis of strawberries (a) and mushrooms (b).

5.2.4.2 Protocol development for image analysis of lettuce

The head of lettuce was conventionally divided into three parts (Figure 22):

1 the side facing up inside the drawer (A);

2 the side facing down in contact with the drawer (B);

3 root area (R).

For each side, eight positions (a, b, c, d, e, f, g, h) were considered turning approximately the lettuce of 45° (Figure 22). The procedure was replicated three times. Only for the first replication, for each side-position three shots were taken in a range of 5 s, waiting for 1 s between one shot and the following. The photos were taken on day 0 and after 7 days of storage at refrigeration temperature (4.0 ± 0.5 °C; 79 ± 12% RH) without treatment.

5.2.5 Color parameters

The processing of the pictures with MATLAB ® software allowed to perform the colorimetric analysis according to the CIE *L*a*b** color system and, therefore, *C**, *H*°, Δ*E*, and *BI* parameters were obtained according to the formulas reported in the section 3.2.4.

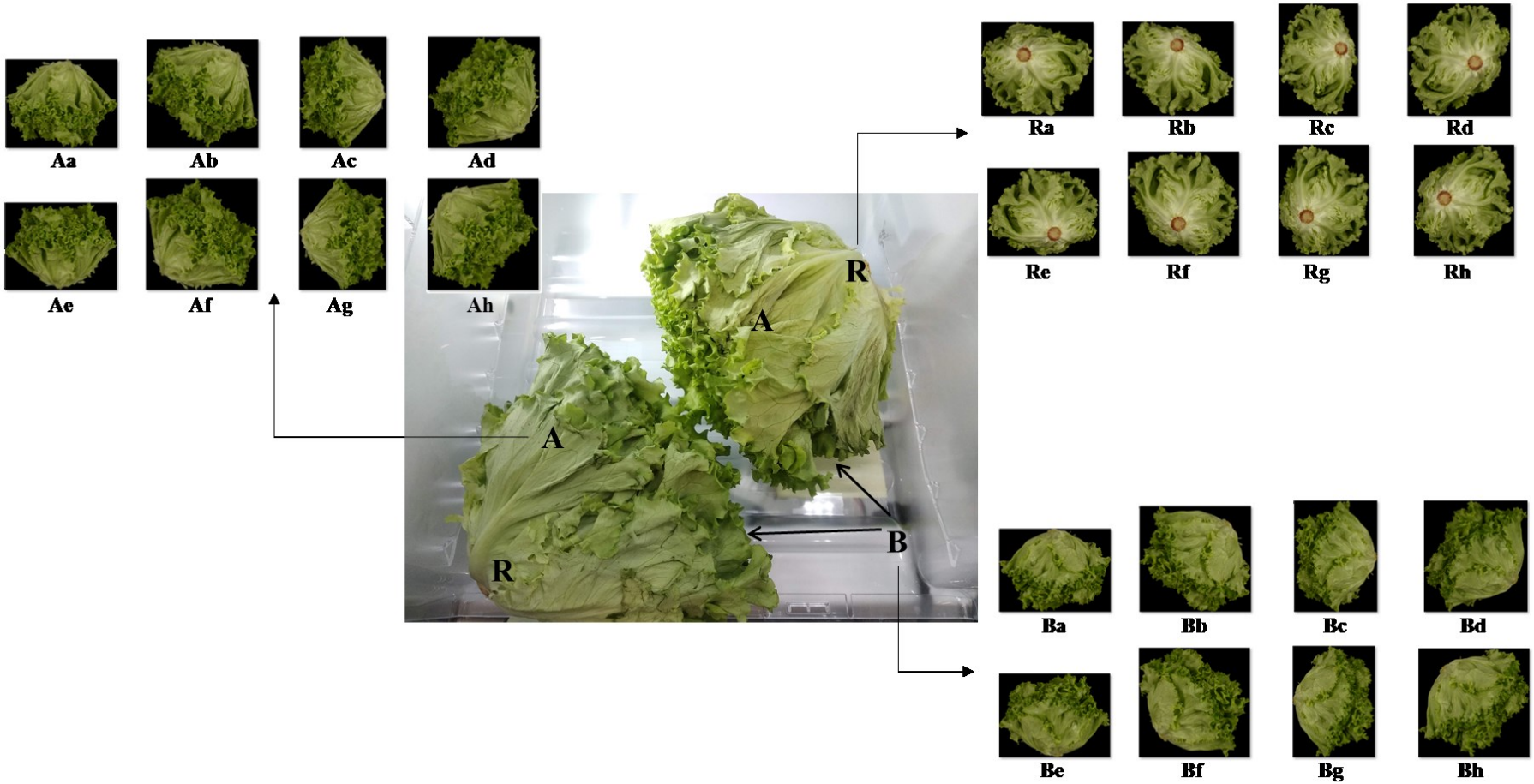

Figure 22 Parts of the lettuce head conventionally used for image analysis: (A) side facing up, (B) side in contact with the drawer, (R) root area. For each side, 8 photos (a, b, c, d, e, f, g, h) were taken turning the lettuce approximately 45°.

5.2.6 Weight loss and shrinkage index

On days 0, 7, and 14 the foods were weighted with a precision balance (ME1002T/00, Mettler Toledo); for each weighing three replicates were made. Weight loss was calculated at days 7 and 14 according to the following formula:

$$W_{loss}\% = \frac{W_0 - W_n}{W_0} \times 100$$

where:

W_0 = weight of the sample at day 0

W_n = weight of the sample at days 7 and 14

The shrinkage index (SI), the effective size reduction of the samples, was calculated with the following formula:

$$SI = 1 - \frac{size_{dayn}}{size_{day0}}$$

where:

$size_{dayn}$ = area or perimeter measured in pixels on days 7 and 14

$size_{day0}$ = area or perimeter measured in pixels on day 0

The number of pixels was a value given by the MATLAB ® tool during the processing of the image.

5.2.7 pH analysis

On days 0, 7, and 14 the foods were subjected to pH analysis. 10 g of foods were crumbled up and blended for 2 min in 10 mL of deionized water. The pH of the suspension was measured at room temperature using a pH meter (Seven Excellence pH meter S400, Mettler Toledo). Three measurements were taken for each sample.

5.2.8 Microbiological analysis

Microbiological analysis was carried out at days 0, 7, and 14 as described in section 3.2.6. Decimal serial dilutions were done and plated on PCA (Oxoid, Milan, Italy) for the total bacterial count and incubated at 30 °C for 24-48 h, and on Violet Red Bile Glucose Agar (VRBG, Oxoid, Milan, Italy) for the enumeration of the *Enterobacteriaceae* and incubated at 37 °C for 24 h.

5.2.9 Statistical analysis

Lettuce, strawberry, and mushroom trials were carried out in at least two biological replicates. For each of the analyses performed, two or three technical repetitions were done. The means obtained from replicate tests were subjected to one-way analysis of variance ($p < 0.05$), preceded by the Levene test to verify the homogeneity of variance between means using Statistics 8.0 (Statsoft software, Tulsa, Oklahoma, USA). Differences between the means were assessed using Tukey's HSD post-hoc test.

5.3 Results and discussion

5.3.1 Protocol development for image analysis of lettuce

To conduct an image analysis that allowed to faithfully follow the progress of food storage over time, the company developed a color imaging system, also called a vision machine. This system was created to have a standardized setting and reproducible surrounding conditions when taking photos. In addition, the analysis is not destructive, therefore image analysis can be done always in the same sample of food during the period of the experimentation avoiding the variability between samples. However, the correct execution of the analysis remained a key issue, so the development of a protocol to carry out an accurate analysis for each type of food was a necessary step.

Lettuce has a heterogeneous and irregular shape, therefore, to obtain an accurate analysis of the image, more than one picture must be taken. The three different sides (A, B, R) aimed to investigate how the position of the lettuce in the drawer influenced the appearance of the parts exposed or not to the air ionization treatment. The eight positions were aimed (a, b, c, d, e, f, g, h) to investigate the variance according to different light/shaded areas. The three shots taken in a range of 5 s aimed to verify the stability of the lighting system. On day 0, the head of the lettuce was uniform in all its parts and the data of the image analysis cannot allow observing any difference during the investigation of the color parameters of the different sides. For that reason, the results presented below referred to the image analysis carried out on day 7 because the positioning of the sample inside the drawer influenced differently the sides of the lettuce.

Step 1. Verification of lighting system stability.

The first step of the protocol development was to verify the stability of the lighting system by taking three shots in a range of 5 s. The results are reported in Table 19. To evaluate the stability of the illumination, the L^* parameter was the most important to consider since it refers to the lightness of the sample. Not being able to perform statistical analysis on single data, the single values of the three shots of the same side-position combination were compared calculating the mean and the standard deviation (SD). An SD value higher than 0.25 was considered as the threshold limit to indicate a variability of lightness. Four combinations (Ab, Ae, Be, Bf) overcame this value, while the others (> 83%) were within the threshold limit. Also, a^* and b^* values and therefore C^* and $H°$ confirmed the poor variability between the three shots, allowing to conclude that the lighting system was stable and that turning on the lamps 30 min before using the vision machine was sufficient time to warm up according to the procedure.

Step 2. Investigation of the light/shaded areas of the lettuce on the color parameters.

The following step of method development was to understand if there was any variability due to different light/shade areas created by the lettuce morphology by rotating eight times the sample approximately 45°. The results, expressed as the mean of three repetitions, are reported in

Table **20** and showed that there was no variability between the eight positions investigated for all the color parameters (L^*, a^*, b^*, C^*, $H°$). Only for the L^* parameter of side B, a difference was observed ($p < 0.05$) between two positions (c and e), while the six remainings were similar ($p > 0.05$). This outcome gave the possibility to reduce the number of positions for each side, allowing to save time for performing the analysis. The number of positions for each side was therefore reduced to four by rotating the lettuce approximately 90° between one position and the following.

Table 19 Results (mean ± SD) of three shots taken into the same side-position combination of lettuce in the range of 5 s for the study of lighting system stability. Sides: A, B, R. Positions: a, b, c, d, e, f, g, h.

		L*	***a****	***b****	***C****	***H°***
A	a	54.03 ± 0.18	-14.28 ± 0.08	36.52 ± 0.11	39.21 ± 0.07	178.80 ± 0.00
	b	53.85 ± 0.85	-14.43 ± 0.12	36.76 ± 0.20	39.49 ± 0.21	178.80 ± 0.00
	c	55.03 ± 0.14	-14.48 ± 0.06	37.11 ± 0.09	39.83 ± 0.06	178.80 ± 0.00
	d	54.71 ± 0.17	-14.53 ± 0.04	37.11 ± 0.01	39.85 ± 0.01	178.80 ± 0.00
	e	52.96 ± 0.26	-14.51 ± 0.04	36.93 ± 0.04	39.68 ± 0.03	178.80 ± 0.00
	f	53.38 ± 0.19	-14.50 ± 0.04	37.04 ± 0.06	39.77 ± 0.04	178.80 ± 0.00
	g	53.54 ± 0.24	-14.46 ± 0.06	36.89 ± 0.08	39.62 ± 0.05	178.80 ± 0.00
	h	53.10 ± 0.16	-14.46 ± 0.05	36.73 ± 0.11	39.47 ± 0.08	178.80 ± 0.00
B	a	53.65 ± 0.13	-13.66 ± 0.08	37.82 ± 0.08	40.21 ± 0.05	178.78 ± 0.00
	b	53.30 ± 0.11	-13.64 ± 0.04	37.81 ± 0.09	40.20 ± 0.07	178.78 ± 0.00
	c	53.41 ± 0.22	-13.60 ± 0.02	37.81 ± 0.08	40.18 ± 0.07	178.77 ± 0.00
	d	52.92 ± 0.08	-13.66 ± 0.05	37.86 ± 0.09	40.25 ± 0.07	178.78 ± 0.00
	e	52.97 ± 0.81	-13.77 ± 0.13	38.10 ± 0.17	40.51 ± 0.20	178.78 ± 0.00
	f	53.42 ± 0.52	-13.66 ± 0.10	38.08 ± 0.25	40.46 ± 0.26	178.77 ± 0.00
	g	53.53 ± 0.18	-13.50 ± 0.05	37.71 ± 0.08	40.05 ± 0.06	178.77 ± 0.00
	h	52.85 ± 0.19	-13.57 ± 0.04	37.66 ± 0.07	40.03 ± 0.05	178.78 ± 0.00
R	a	45.77 ± 0.15	-7.98 ± 0.10	31.69 ± 0.01	32.68 ± 0.10	178.68 ± 0.00
	b	46.97 ± 0.14	-8.16 ± 0.10	32.16 ± 0.04	33.18 ± 0.10	178.68 ± 0.00
	c	46.31 ± 0.09	-8.36 ± 0.04	32.55 ± 0.04	33.61 ± 0.03	178.68 ± 0.00
	d	45.69 ± 0.08	-8.35 ± 0.06	32.11 ± 0.03	33.18 ± 0.05	178.68 ± 0.00
	e	45.60 ± 0.13	-8.15 ± 0.04	31.92 ± 0.03	32.94 ± 0.04	178.68 ± 0.00
	f	44.93 ± 0.11	-8.17 ± 0.04	32.30 ± 0.02	33.32 ± 0.03	178.68 ± 0.00
	g	44.81 ± 0.13	-8.18 ± 0.04	32.32 ± 0.02	33.34 ± 0.04	178.68 ± 0.00
	h	45.13 ± 0.14	-8.03 ± 0.02	31.88 ± 0.04	32.88 ± 0.03	178.68 ± 0.00

Table 20 Results (mean ± SD) of three repetitions done for each side-position combination of lettuce. Sides: A, B, R. Positions: a, b, c, d, e, f, g, h.

		*L**	*a**	*b**	*C**	*H°*
A	a	52.70 ± 1.84 [a*]	-14.33 ± 0.49 [a]	36.85 ± 0.09 [a]	39.54 ± 0.50 [a]	178.80 ± 0.00 [a]
	b	52.79 ± 1.64 [a]	-14.44 ± 0.45 [a]	37.03 ± 0.09 [a]	39.75 ± 0.47 [a]	178.80 ± 0.00 [a]
	c	53.36 ± 2.41 [a]	-14.45 ± 0.18 [a]	37.25 ± 0.06 [a]	39.95 ± 0.21 [a]	178.80 ± 0.00 [a]
	d	53.09 ± 2.31 [a]	-14.45 ± 0.16 [a]	37.26 ± 0.11 [a]	39.96 ± 0.21 [a]	178.80 ± 0.00 [a]
	e	51.46 ± 2.11 [a]	-14.45 ± 0.20 [a]	37.05 ± 0.12 [a]	39.77 ± 0.22 [a]	178.80 ± 0.00 [a]
	f	51.90 ± 2.07 [a]	-14.48 ± 0.21 [a]	37.15 ± 0.09 [a]	39.87 ± 0.21 [a]	178.80 ± 0.00 [a]
	g	52.26 ± 1.89 [a]	-14.40 ± 0.33 [a]	37.10 ± 0.11 [a]	39.80 ± 0.37 [a]	178.80 ± 0.01 [a]
	h	51.89 ± 1.74 [a]	-14.41 ± 0.29 [a]	36.93 ± 0.11 [a]	39.64 ± 0.32 [a]	178.80 ± 0.00 [a]
B	a	53.32 ± 0.48 [ab]	-13.42 ± 0.26 [a]	37.75 ± 0.35 [a]	40.06 ± 0.16 [a]	178.77 ± 0.01 [a]
	b	53.22 ± 0.13 [ab]	-13.38 ± 0.20 [a]	37.80 ± 0.37 [a]	40.10 ± 0.12 [a]	178.77 ± 0.01 [a]
	c	53.47 ± 0.22 [a]	-13.33 ± 0.16 [a]	37.79 ± 0.37 [a]	40.08 ± 0.08 [a]	178.77 ± 0.01 [a]
	d	52.79 ± 0.18 [ab]	-13.40 ± 0.17 [a]	37.83 ± 0.37 [a]	40.14 ± 0.09 [a]	178.77 ± 0.01 [a]
	e	52.54 ± 0.83 [b]	-13.48 ± 0.36 [a]	37.96 ± 0.41 [a]	40.29 ± 0.26 [a]	178.77 ± 0.01 [a]
	f	53.15 ± 0.52 [ab]	-13.41 ± 0.38 [a]	37.96 ± 0.36 [a]	40.26 ± 0.30 [a]	178.77 ± 0.01 [a]
	g	53.40 ± 0.29 [ab]	-13.30 ± 0.15 [a]	37.72 ± 0.28 [a]	39.99 ± 0.11 [a]	178.77 ± 0.01 [a]
	h	52.76 ± 0.30 [ab]	-13.34 ± 0.17 [a]	37.66 ± 0.32 [a]	39.95 ± 0.12 [a]	178.77 ± 0.01 [a]
R	a	45.19 ± 0.85 [a]	-8.05 ± 0.27 [a]	31.85 ± 0.10 [a]	32.85 ± 0.25 [a]	178.68 ± 0.00 [a]
	b	45.82 ± 1.58 [a]	-8.19 ± 0.19 [a]	32.07 ± 0.06 [a]	33.09 ± 0.19 [a]	178.68 ± 0.00 [a]
	c	45.83 ± 0.75 [a]	-8.44 ± 0.29 [a]	32.59 ± 0.17 [a]	33.67 ± 0.26 [a]	178.68 ± 0.00 [a]
	d	45.44 ± 0.41 [a]	-8.43 ± 0.33 [a]	32.31 ± 0.17 [a]	33.39 ± 0.30 [a]	178.68 ± 0.00 [a]
	e	44.98 ± 0.91 [a]	-8.23 ± 0.16 [a]	31.99 ± 0.15 [a]	33.04 ± 0.12 [a]	178.68 ± 0.00 [a]
	f	44.45 ± 0.77 [a]	-8.25 ± 0.17 [a]	32.23 ± 0.13 [a]	33.27 ± 0.17 [a]	178.68 ± 0.00 [a]
	g	44.28 ± 0.73 [a]	-8.24 ± 0.10 [a]	32.31 ± 0.10 [a]	33.34 ± 0.09 [a]	178.68 ± 0.00 [a]
	h	44.36 ± 1.15 [a]	-8.10 ± 0.13 [a]	31.92 ± 0.10 [a]	32.93 ± 0.13 [a]	178.68 ± 0.00 [a]

* For each side and color parameter, means with different letters within a column are significantly different ($p < 0.05$).

Step 3. Investigation of the different sides of the lettuce sample.

The last step of the protocol development for the image analysis of lettuce was the investigation of the differences between the sides conventionally identified in the sample: A for the side facing up in the drawer, B for the side facing down directly in contact with the drawer, and R for the root area. The results are shown in Table 21. For all the color parameters considered in the analysis, there was a significant difference ($p < 0.05$) between the three sides, indicating that the different parts of the lettuce were affected differently during the conservation at refrigeration temperature inside the crisper. Therefore, the different parts of the lettuce must be investigated to obtain a representative final value of color change (ΔE).

Table 21. Results (mean ± SD) of the eight positions investigated for each side (A, B, R).

	L*	***a****	***b****	***C****	***H°***
A	52.43 ± 1.93 [a*]	-14.43 ± 0.32 [a]	37.08 ± 0.10 [b]	39.79 ± 0.33 [b]	178.80 ± 0.00 [a]
B	53.08 ± 0.50 [a]	-13.38 ± 0.25 [b]	37.81 ± 0.33 [a]	40.11 ± 0.19 [a]	178.77 ± 0.01 [b]
R	45.05 ± 1.05 [b]	-8.24 ± 0.32 [c]	32.16 ± 0.18 [c]	33.20 ± 0.30 [c]	178.68 ± 0.00 [c]

* For each color parameter, means with different letters within a column are significantly different ($p < 0.05$).

Summarizing, the protocol for the image analysis of lettuce had established the following procedure:

1 Investigate the three sides A, B, and R conventionally identified for the head of lettuce
2 Take four pictures (a, b, c, d,) for each side by rotating the lettuce approximately 90°
3 Calculate the color parameters by taking the average of the values obtained by processing all the photos (as for mushroom and strawberry protocol)

5.3.2 Image analysis

The results of the image analysis of lettuce, strawberries, and mushrooms are reported in Table 22. *L**, *a*, b** values were obtained directly from the analysis of the images with the MATLAB ® software that gave the color parameters according to the CIE *L*a*b** color systems; *C**, *H°*, Δ*E*, and *BI* were calculated from *L**, *a*,* and *b** values. In general, most of the results showed that the color parameters did not change significantly during the14 days of the ionization treatment and compared to the relative controls. Lettuce resulted to be the food with the lowest impact of the treatment, showing to have a total color change lower than control samples after 14 days (Figure 23). This outcome was very important considering that the visual appearance of food has a primary role in the acceptability of consumers.

Table 22 Color parameters (L^*, a^*, b^*, C^*, $H°$, ΔE, and BI; mean ± SD) obtained by processing the images with MATLAB ® software.

		Lettuce		**Strawberry**		**Mushroom**	
	day	Control	Control	Treated	Treated	Control	Treated
L*	0	41.49 ± 5.18 ^{aA*}	21.28 ± 2.39 ^{aA}	20.91 ± 3.09 ^{aA}	41.89 ± 3.57 ^{aA}	62.04 ± 3.81 ^{aA}	62.47 ± 4.28 ^{aA}
	7	44.23 ± 4.73 ^{aA}	21.06 ± 2.67 ^{aA}	20.20 ± 2.96 ^{aA}	44.43 ± 4.12 ^{aA}	60.04 ± 7.71 ^{aA}	55.34 ± 5.04 ^{aA}
	14	44.84 ± 4.58 ^{aA}	20.05 ± 2.38 ^{aA}	18.01 ± 2.41 ^{aA}	44.50 ± 5.18 ^{aA}	57.95 ± 7.73 ^{aA}	53.78 ± 5.71 ^{aA}
a*	0	-14.58 ± 2.07 ^{aA}	29.84 ± 2.85 ^{aA}	28.77 ± 3.63 ^{aA}	-14.55 ± 1.82 ^{aA}	-1.15 ± 0.91 ^{aA}	-1.13 ± 1.02 ^{aA}
	7	-12.88 ± 1.66 ^{aA}	28.76 ± 3.33 ^{aA}	26.52 ± 3.57 ^{aA}	-12.84 ± 1.50 ^{aA}	-1.43 ± 1.10 ^{aA}	0.29 ± 0.80 ^{aA}
	14	-12.13 ± 1.87 ^{aA}	25.52 ± 3.25 ^{aA}	22.08 ± 2.44 ^{aA}	-12.17 ± 1.53 ^{aA}	-0.12 ± 1.55 ^{aA}	0.95 ± 1.15 ^{aA}
b*	0	36.60 ± 2.38 ^{aA}	24.78 ± 3.43 ^{aA}	23.39 ± 3.41 ^{aA}	36.80 ± 2.08 ^{aA}	20.20 ± 2.95 ^{aA}	20.18 ± 3.76 ^{bA}
	7	35.34 ± 1.60 ^{aA}	23.40 ± 3.56 ^{aA}	21.14 ± 3.37 ^{aA}	35.51 ± 1.52 ^{aA}	25.25 ± 1.80 ^{aB}	28.19 ± 1.15 ^{aA}
	14	35.03 ± 1.93 ^{aA}	20.84 ± 3.30 ^{aA}	17.90 ± 2.58 ^{aA}	35.08 ± 1.56 ^{aA}	27.05 ± 1.63 ^{aA}	28.85 ± 1.27 ^{aA}
C*	0	39.43 ± 2.79 ^{aA}	38.79 ± 4.35 ^{aA}	37.08 ± 4.94 ^{aA}	39.59 ± 2.49 ^{aA}	20.26 ± 2.92 ^{bA}	20.24 ± 3.73 ^{bA}
	7	37.63 ± 2.00 ^{aA}	37.09 ± 4.79 ^{aA}	33.92 ± 4.87 ^{abA}	37.77 ± 1.87 ^{aA}	25.31 ± 1.75 ^{abA}	28.20 ± 1.14 ^{aA}
	14	37.09 ± 2.35 ^{aA}	32.96 ± 4.51 ^{aA}	28.44 ± 3.38 ^{bA}	37.14 ± 1.93 ^{aA}	27.09 ± 1.62 ^{aA}	28.88 ± 1.27 ^{aA}
H°	0	178.81 ± 0.04 ^{aA}	0.69 ± 0.03 ^{aA}	0.68 ± 0.02 ^{aB}	178.80 ± 0.03 ^{aA}	178.66 ± 0.72 ^{aA}	179.01 ± 1.16 ^{aA}
	7	178.78 ± 0.03 ^{aA}	0.68 ± 0.03 ^{aA}	0.67 ± 0.02 ^{aB}	178.78 ± 0.03 ^{aA}	179.01 ± 1.17 ^{aA}	180.51 ± 1.50 ^{aA}
	14	178.76 ± 0.04 ^{aA}	0.68 ± 0.04 ^{aA}	0.68 ± 0.04 ^{aA}	178.76 ± 0.03 ^{aA}	179.83 ± 1.56 ^{aA}	180.84 ± 1.31 ^{aA}
ΔE	0	-	-	-	-	-	-
	7	2.63 ± 0.74 ^{aA}	2.43 ± 1.52 ^{aA}	3.29 ± 0.92 ^{aA}	2.59 ± 0.29 ^{bA}	7.25 ± 1.99 ^{aB}	11.45 ± 1.93 ^{aA}
	14	4.92 ± 1.90 ^{aA}	6.10 ± 3.12 ^{aB}	9.00 ± 2.00 ^{aA}	3.99 ± 0.79 ^{aA}	9.62 ± 2.35 ^{aB}	13.18 ± 1.74 ^{aA}
BI	0	-	-	-	-	-	-
	7	-	-	-	-	0.38 ± 0.07 ^{aB}	0.64 ± 0.23 ^{aA}
	14	-	-	-	-	0.87 ± 0.09 ^{aA}	1.04 ± 0.17 ^{aA}

*For each color parameter of the same food: means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$); means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

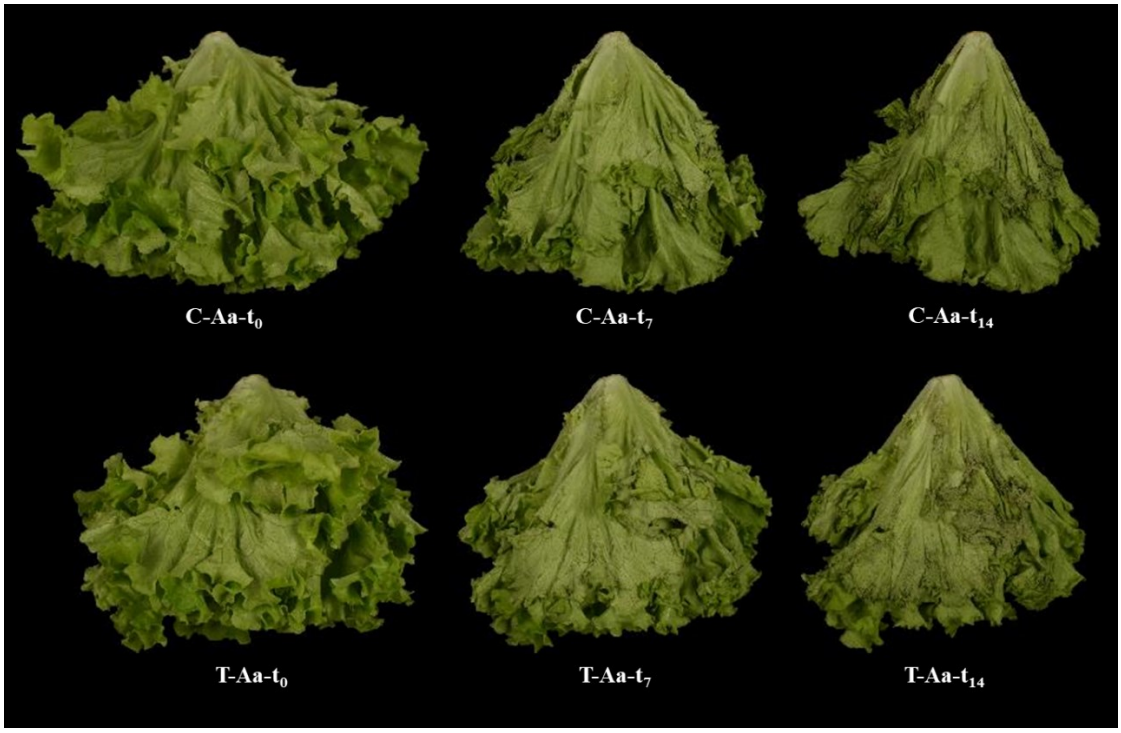

Figure 23 Example of lettuce image analysis. Comparison of the same control (C) and treated (T) lettuce on day 0 (t_0), day 7 (t_7), and day 14 (t_{14}). Images were taken in the Aa side-position combination.

Only a few results were observed to change significantly ($p < 0.05$), particularly for strawberries and mushrooms. In the case of strawberries, the C^* value of the treated samples decreased during the 14 days of exposure to ionization, indicating a decrease in the color saturation of the samples and $H°$ value was significantly lower ($p < 0.05$) in the treated than control samples, suggesting a loss in the hue color. The difference between the two samples was confirmed by the ΔE value at the end of the 14 days, which is significantly higher ($p < 0.05$) for the treated strawberries. Figure 24 highlights the outcomes that emerged with the color parameters analysis, showing how the loss of hue in treated fruits increased during the days. These findings on strawberries contrasted with other studies that involved the use of similar technologies. The use of gaseous ozone (0.5 – 1.5 ppm) for 40 min was investigated by Panou et al. (2021) to improve the quality of strawberries under the following storage at refrigerated conditions (1 C° and 90% RH) for 15 days. They found that the treatment of strawberries at a low dose (0.5 ppm) extended the shelf-life of the fruits, but the use of high doses accelerated the deterioration in appearance. Another investigation was done by using the cold plasma treatment for a maximum of 20 min, demonstrating that it had no negative effect on the fruit color and that the plasma-treated samples were better in

quality than the controls at the end of 12 days of storage at 6 °C (Ahmadnia et al., 2021). However, even if DM used for the ionization tests on food generated NAI and a low concentration of ozone (0.02 mg/h), probably it affected the quality of the treated strawberries because it was used in a continuous mode for all the duration of the tests and the constant presence of oxidative species negatively influenced the quality of the food. For mushrooms, the b^* parameter, which is related to the degree of yellowness ($+b^*$) or blueness ($-b^*$), indicated a yellowing of the treated samples after 7 days. C^* value increased during the 14 days both in the control and treated samples suggesting an increase of color saturation. The values of ΔE indicated that for the treated samples the total color change was higher than the control samples after 7 and 14 days, while the *BI* indicated that the browning was higher in the treated samples after 7 days, but that after 14 days it was similar ($p > 0.05$) in both control and treated mushrooms. The phenomenon of browning, the change of color from white to brown, is common in mushrooms after harvest and it is due to enzymatic oxidation, senescence, and microbial growth (Lin & Sun, 2019). In the control samples, the browning was heterogenous with the presence of portions more browned than others in the cap or stipe samples (Figure 25). During the ionization treatment, a higher homogeneous browning was observed in the treated mushrooms because the presence of oxidative active species as NAI and ozone accelerated the enzymatic browning reaction (Escriche et al., 2001).

The color vision machine represented an effective method to perform colorimetric analysis on food samples. The analysis was not destructive, therefore image analysis can be done always in the same sample of food during the period of the experimentation avoiding the variability between samples, a critical aspect emerged during the color determination in the previous study of ionization technology on food; section 3.4.

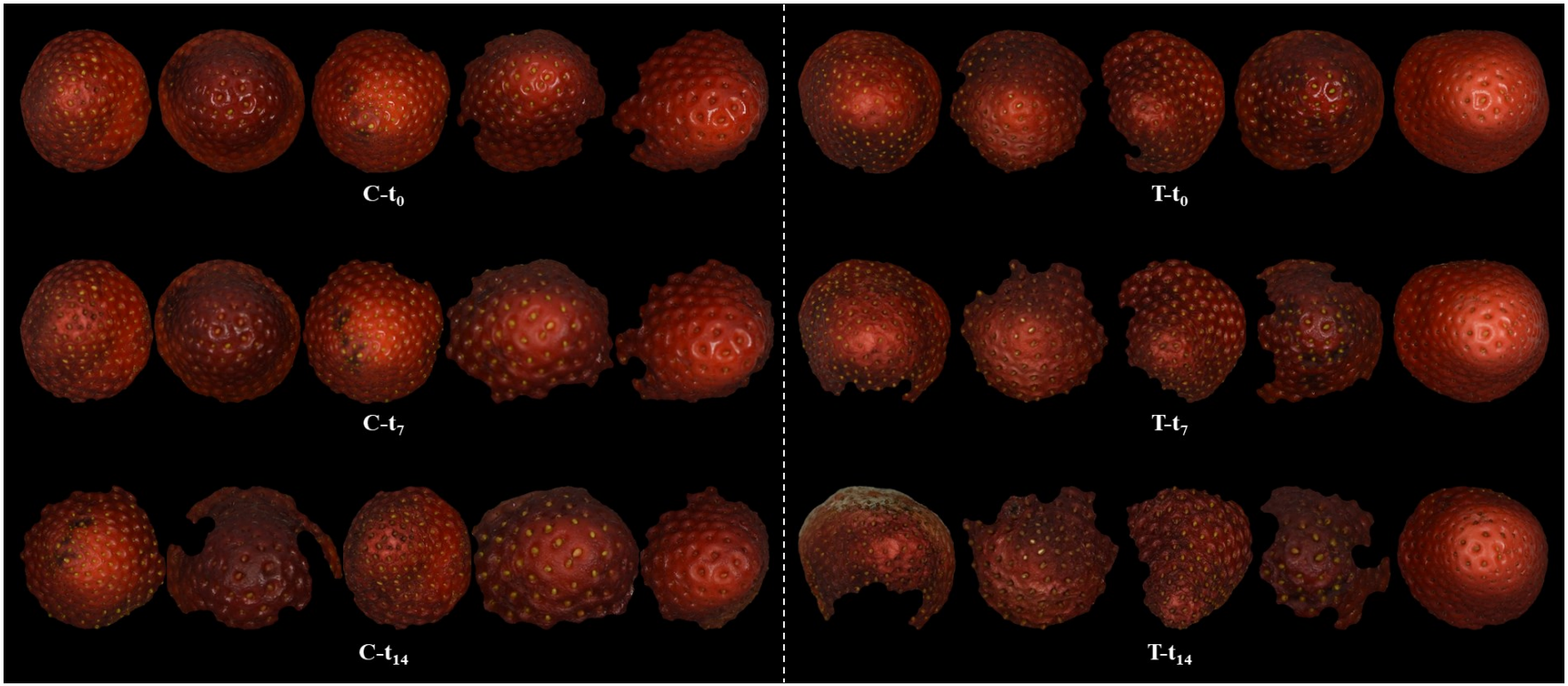

Figure 24 Example of strawberry image analysis. Comparison between control (C) and treated (T) fruits on day 0 (t_0), day 7 (t_7), and day 14 (t_{14}).

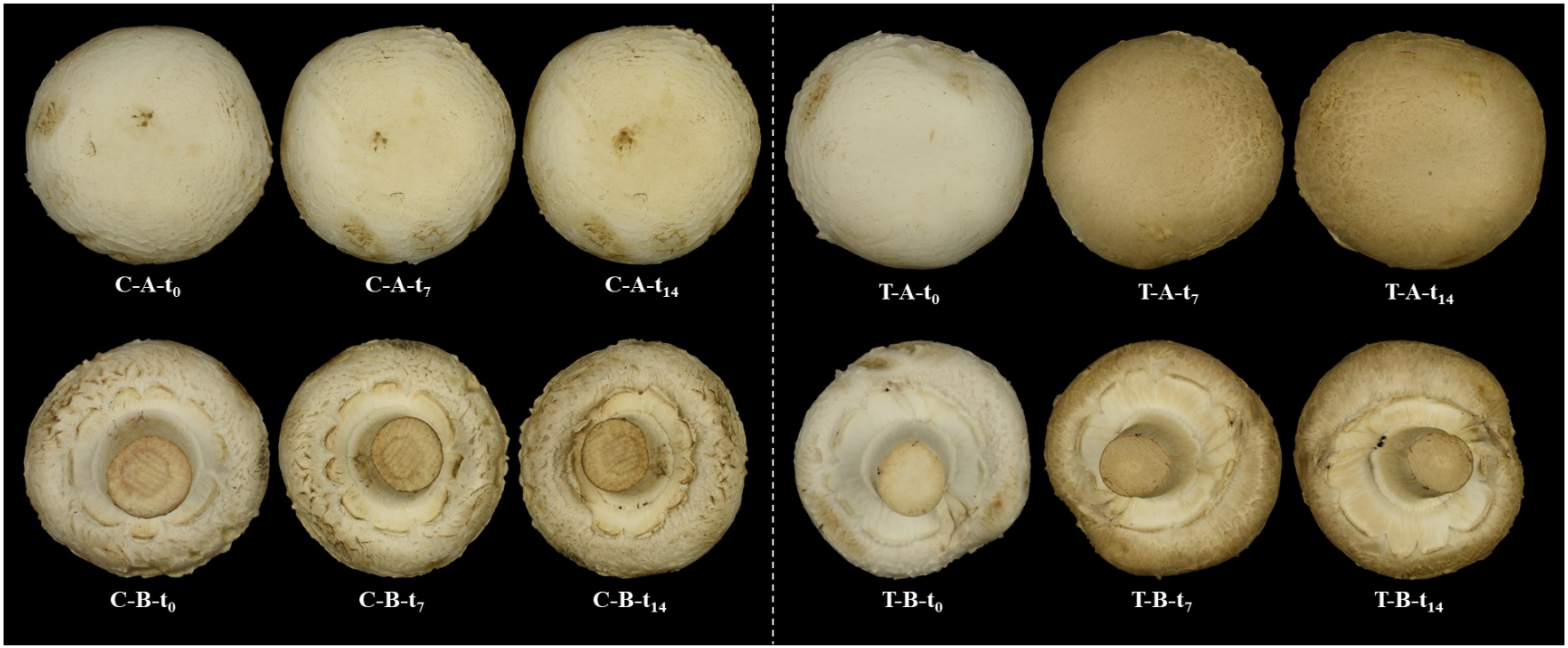

Figure 25 Example of mushroom image analysis. Comparison of the same control (C) and treated (T) mushrooms in the cap (A) and stipe (B) positions on day 0 (t_0), day 7 (t_7), and day 14 (t_{14}).

5.3.1 Weight loss, shrinkage index, and correlation between weight and image size

The weight loss % of lettuce, mushrooms, and strawberries is reported in Figure 26. In general, the weight loss increased significantly ($p < 0.05$) increasing the period of the storage for both control and treated samples of all the foods tested. In addition, the foods exposed to ionization treatment showed no significant difference ($p > 0.05$) compared to the control samples in the case of lettuce and mushrooms. Lettuce weight loss was 15 ± 4% and 26 ±5% in the control samples and 16 ± 2% and 26 ±3% in the treated samples after 7 and 14 days respectively. Strawberry weight loss was 10 ± 1% and 21 ±1% in the control samples and 13 ± 1% and 28 ±1% in the treated samples after 7 and 14 days respectively. Mushroom weight loss was 9 ± 2% and 18 ±4% in the control samples and 12 ± 3% and 25 ±7% in the treated samples after 7 and 14 days respectively. The loss of weight is mainly due to the water transpiration and CO_2 loss during respiration and represents a normal behavior during the storage of fruits and vegetables. The rate of water loss depends on respiration rate, fluctuations of temperature, and relative humidity (Kurubas et al., 2019; Panou et al., 2021). Considering the results obtained comparing the control and treated samples, it was possible to conclude that the treatment with ionization technology did not affect the reparation rate and therefore the weight loss of lettuce and mushrooms but influenced those of strawberries. A possible explanation of this result is that ions form clusters with the water molecule in the air when the value of relative humidity is high (79 ± 12% RH inside the drawers) (Schiessling et al., 2018). Consequently, since the fruit of strawberry is smaller than a head of lettuce or a single mushroom, the rate of transpiration is faster in the presence of NAI.

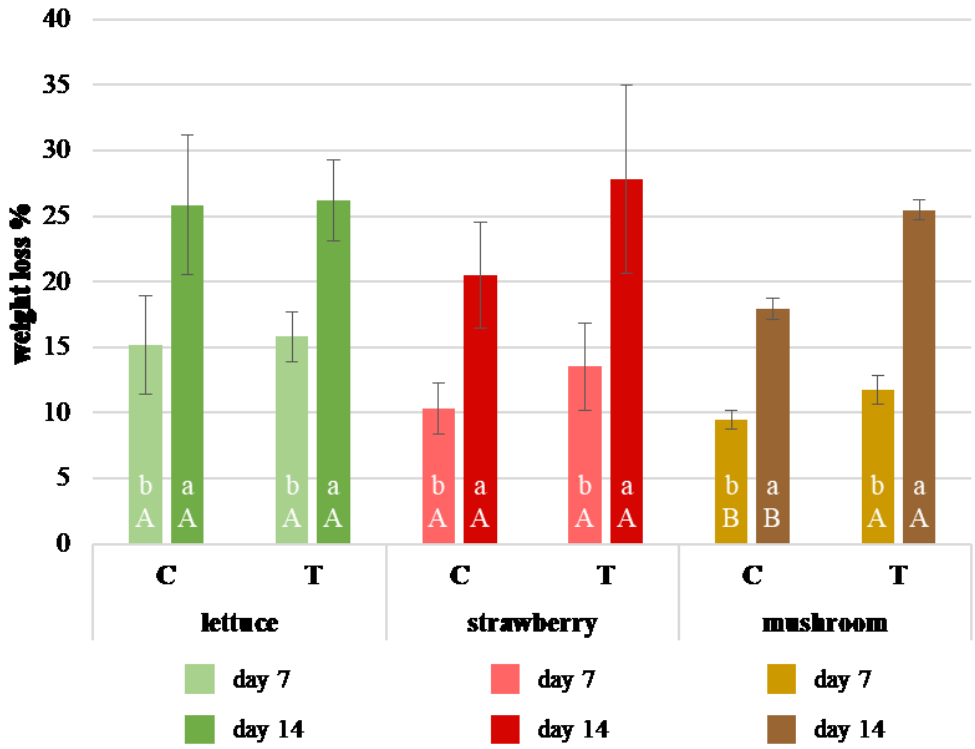

Figure 26 Weigh loss % of control (C) and treated (T) lettuce, mushrooms, and strawberries after 7 and 14 days. C = control sample, T = treated sample. Means with different letters within the food on the same day (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within the sample for the same food (lowercase letter) are significantly different ($p < 0.05$).

Another parameter evaluated was the shrinkage index (*SI*) that is the effective size reduction of the samples calculated by using the pixels of the images. The pixels are correlated with the size of the image obtained after the segmentation, which is the process that precisely identifies the borders of the image (for that reason a matte black soft pad is used for taking pictures). The results of the *SI* are reported in Table 23.

Table 23 Shrinkage index (mean ± SD; n = 3) of lettuce, mushroom, and strawberry.

		Control	Treated
Lettuce	day 7	0.15 ± 0.09 ^{aA*}	0.13 ± 0.06 ^{aA}
	day 14	0.23 ± 0.05 ^{aA}	0.17 ± 0.07 ^{aA}
Strawberry	day 7	0.05 ± 0.12 ^{aA}	0.14 ± 0.08 ^{aA}
	day 14	0.25 ± 0.13 ^{aA}	0.35 ± 0.09 ^{bA}
Mushroom	day 7	0.16 ± 0.33 ^{aA}	0.15 ± 0.21 ^{aA}
	day 14	0.21 ± 0.36 ^{aA}	0.35 ± 0.36 ^{aA}

*For each food, means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$); means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

According to the ANOVA analysis, no significant differences ($p > 0.05$) were observed in any of the tested food between the control and the treated samples, indicating that the ionization treatment did not influence the size of lettuce, mushrooms, and strawberries. A similar shrinkage correlation study was conducted by Vallespir et al. (2019) to evaluate the effect of mushroom drying at low temperatures. Taking an example from their study, the correlation between weight and image size was done (Figure 27). The trends showed that there is a correlation between the weight of the control and treated foods and the reduction of the image size during the 14 days. To have a confirmation of this graphical observation, experimental results of weight (g) and image size (pixels) were fitted to linear regression, and the equations reported in Table 24 were obtained.

Table 24 Linear regression equations, R^2, and ρ obtained fitting weight and image size of lettuce, strawberry, and mushroom.

		Equation of linear regression	**R^2**	**ρ**
Lettuce	Control	$y = 16696x + 2e^6$	0.9969	1.00
	Treated	$y = 12732x + 3e^6$	0.9990	1.00
Strawberry	Control	$y = 2691.5x - 59821$	0.8893	0.94
	Treated	$y = 2599.2x - 72237$	0.9899	0.99
Mushroom	Control	$y = 74044x - 2e^6$	0.9150	0.95
	Treated	$y = 53138x - 70331$	0.8656	0.93

All the linear regressions presented a high correlation coefficient (ρ) close to the units, therefore the linear adjustment was considered satisfactory. The results of the linear regression and the correlation suggested that the image size can be used to estimate the weight, and therefore the weight loss, of lettuce, mushrooms, and strawberry using the color imaging system without the use of a scale. Having achieved this information could allow to reduce the manipulation of the food during experimentations and to decrease the time of the analysis, both positive aspects during the execution of a trial.

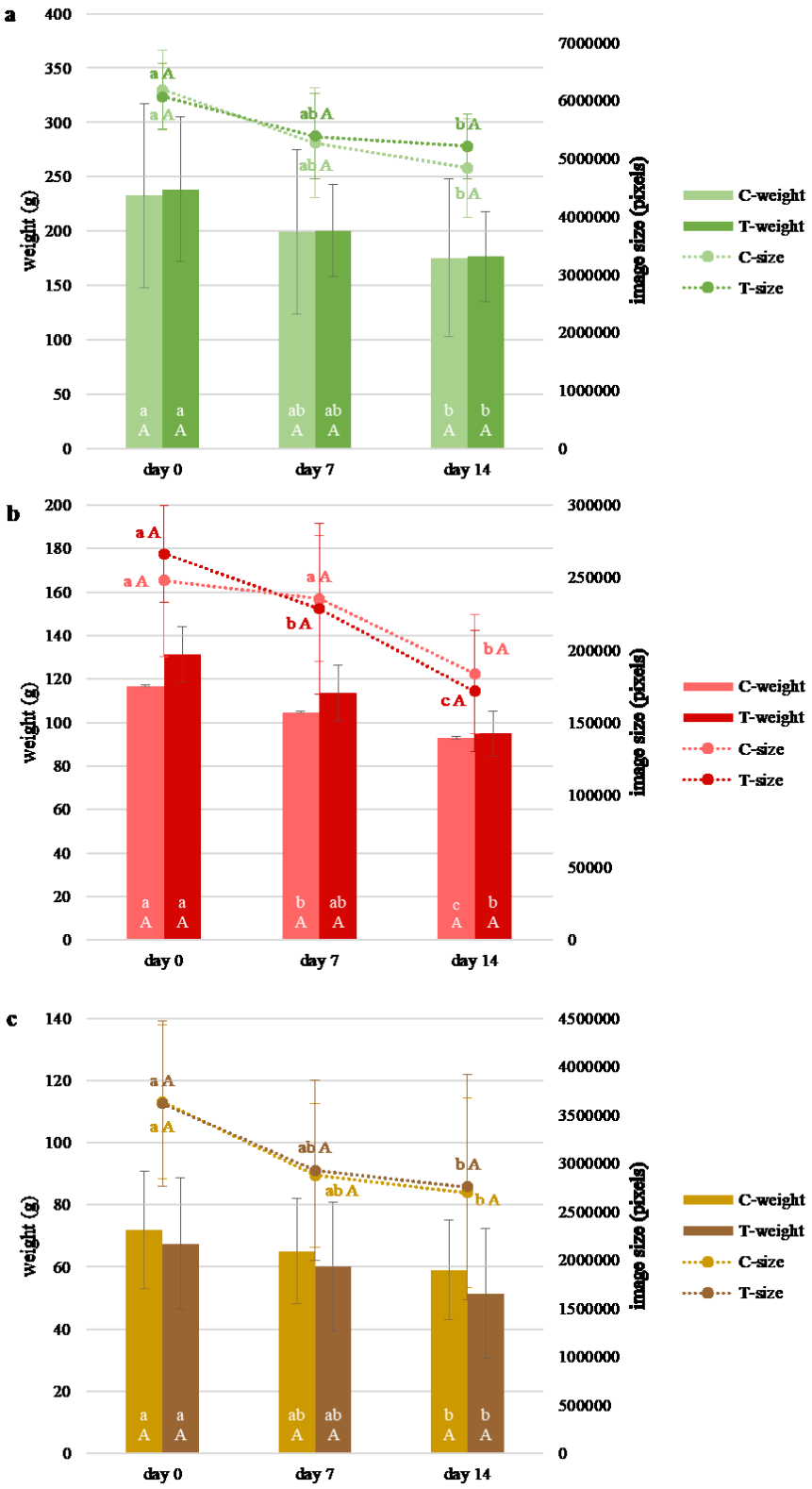

Figure 27 Correlation between weight and image size of lettuce (a), strawberries (b), and mushrooms (c). Means with different letters within the day (uppercase letter) are significantly different ($p < 0.05$). Means with different letters within the sample (lowercase letters) are significantly different ($p < 0.05$).

5.3.2 pH analysis

The pH values of lettuce, mushrooms, and strawberries are reported in Table 25. This physical parameter was investigated because for a technology similar to ionization, the cold plasma (ionized gas that contains a variety of active electrically charged particles as electrons, ions, radicals, and metastable excited species) was observed a decrease in the pH values during the treatments of foods that is probably due to the interaction between water molecules and ions leading to the formation of hydronium ions (H_3O^+) (Muhammad et al., 2018). Only for treated mushrooms, this behavior was observed, with the significant ($p < 0.05$) decrease of the pH after 7 and 14 days of ionization treatment. However, a similar result was observed for the control mushroom and there was no difference comparing the two samples after 7 and 14 days. No significant differences ($p > 0.05$) were observed for lettuce and strawberries. Considering these results, ionization treatment resulted to not affect the pH of the foods during the 14 days of the exposition.

Table 25 pH values (mean ± SD) of lettuce, strawberry, and mushrooms.

	Lettuce		**Strawberry**		**Mushroom**	
day	Control	Treated	Control	Treated	Control	Treated
0	5.91 ± 0.08 ^{aA*}	5.94 ± 0.12 ^{aA}	3.77 ± 0.02 ^{bA}	3.67 ± 0.08 ^{aA}	6.92 ± 0.15 ^{aA}	6.77 ± 0.05 ^{aB}
7	6.01 ± 0.10 ^{aA}	5.93 ± 0.12 ^{aA}	3.92 ± 0.18 ^{aA}	3.74 ± 0.00 ^{aA}	6.66 ± 0.06 ^{bA}	6.61 ± 0.07 ^{bA}
14	5.99 ± 0.11 ^{aA}	6.00 ± 0.07 ^{aA}	3.57 ± 0.48 ^{abA}	3.64 ± 0.14 ^{aA}	6.57 ± 0.06 ^{bA}	6.51 ± 0.04 ^{cA}

*For each food, means with different letters within a row (uppercase letter) are significantly different ($p < 0.05$); means with different letters within a column (lowercase letter) are significantly different ($p < 0.05$).

5.3.3 Microbiological analysis

The results of the microbiological analysis are reported in Figure 29. Total bacterial count (TBC) and *Enterobacteriaceae* were investigated as spoilage evolution and hygienic status indicators respectively. The *Enterobacteriaceae* counts were unvaried for all the foods both in control and treated samples and in some cases was lower than the limit of the method (1 Log CFU/g). The high SD present in some cases was due to the variability of the food samples since the microbiological assay was a destructive analysis and the samples were different each time despite coming from the same batch.

The initial TBC of lettuce was around 6 Log CFU/g for both controls and treated samples and remained stable during the period of the treatment without any significant difference. The TBC count of mushrooms was 6.67 ± 0.73 Log CFU/g and 5.79 ± 0.58 Log CFU/g of control and treated samples respectively. In this case, the counts increased over the 14 days in both samples without any significant difference between the control and the treated mushrooms, confirming the trend observed in the previous treatment (sections 3.3.1.2 and 3.3.2.2). The microbial spoilage on the mushroom is commonly associated with fluorescent pseudomonads causing brown blotch disease that generally begins as surfaces discoloration on caps and stipes for developing into dark-brown, sunken, or pitted lesions (Wells et al., 1996) as illustrated in Figure 28.

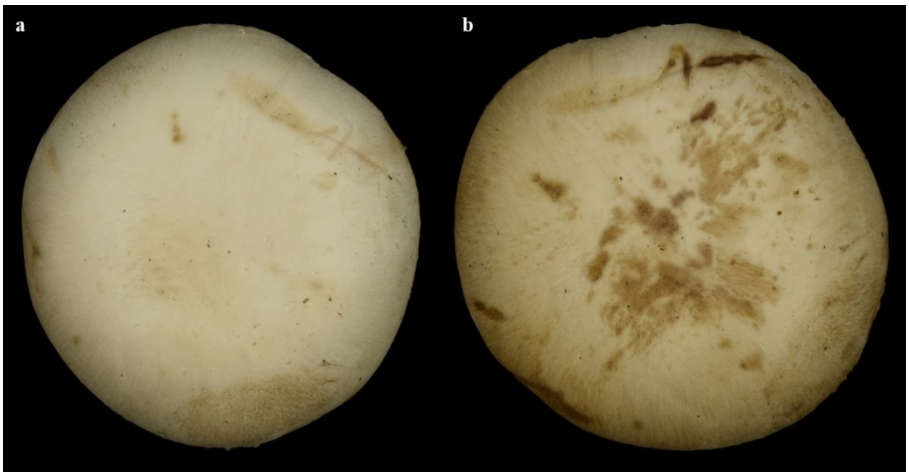

Figure 28 Example of the evolution from day 0 (a) to day 14 (b) of microbial spoilage in a control mushroom.

The TBC count of strawberries was 2.64 ± 0.91 Log CFU/g and 3.17 ± 1.44 Log CFU/g of control and treated samples respectively. A significant ($p < 0.05$) increase during the 14 days was observed for control samples but not for the treated. However, no significant differences were observed between the control and treated strawberries for any of the days considered. However, the results of an investigation with cold plasma for 20 min showed the ability to reduce significantly the microbial populations of TBC and molds (Ahmadnia et al., 2021), while in the present investigation, in one of the biological replications, a microbiological deterioration occurred in one fruit due to the growth of a mold on the surface (Figure 24). Probably, to achieve significant results in terms of microbial reduction the treatment should be stronger, with the risk to negatively affect other qualities related to the acceptability of the product.

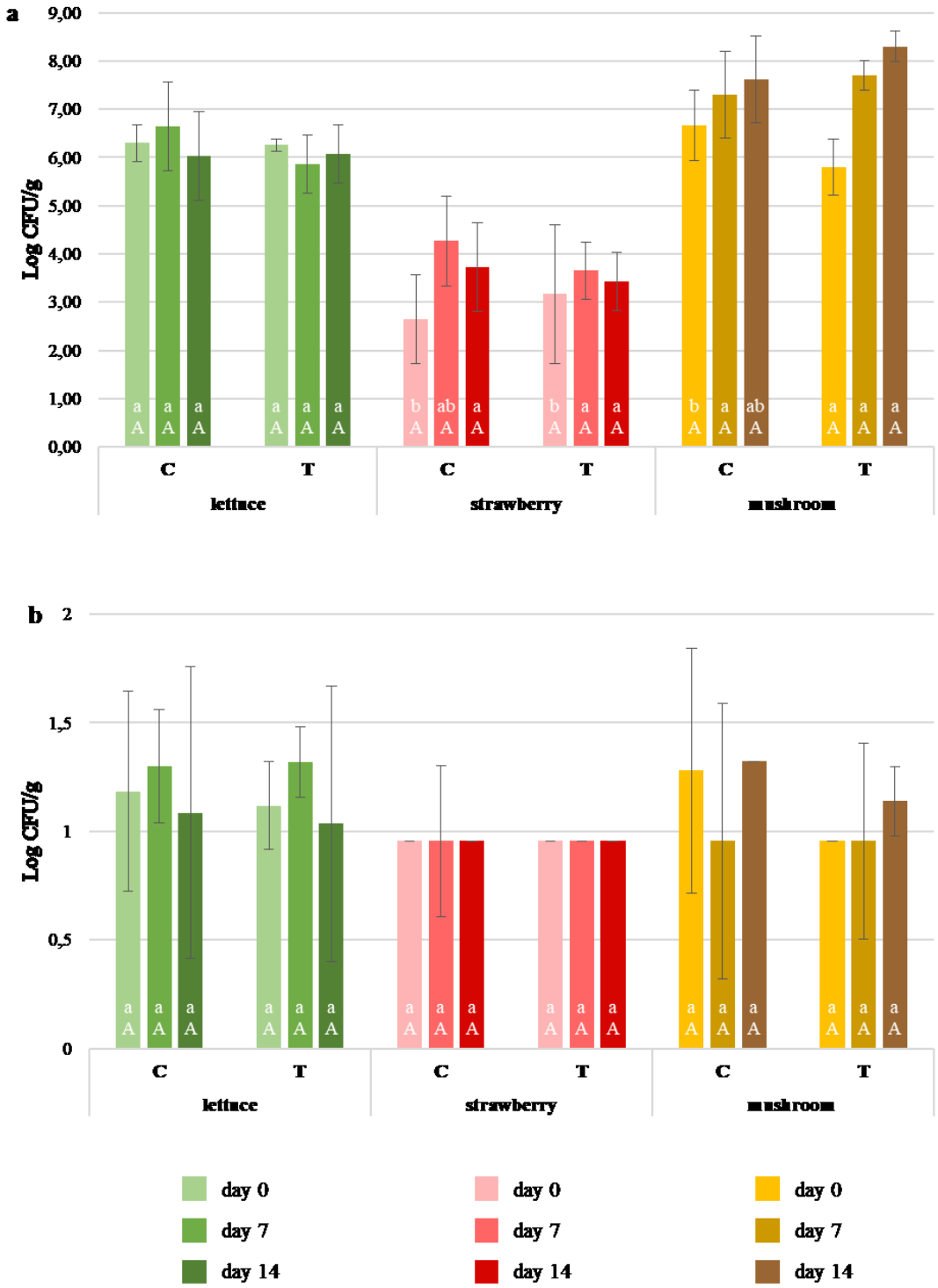

Figure 29 Effect of ionization treatments on the total bacterial count (a) and *Enterobacteriaceae* (b) of lettuce, strawberries, and mushrooms. C = control sample; T = treated sample. Uppercase letters compare control and treated samples of the same food on the same day. Lowercase letters compare the same sample on the different days. Means with different letters are significantly different ($p < 0.05$).

5.4 Considerations about the analysis methodology

The color imaging system resulted in a better method to carry out the analysis of the color than the method used in the previous study for the evaluation of the effect of ionization technology on food (section 3.2.4). The vision system allowed to conduct a non-destructive color determination and the color parameters referred to the whole sample analyzed and not only to few spots, therefore the results obtained were more representative of the real situation.

5.5 Conclusions

The vision machine implemented by the company resulted to be a valid method to perform an accurate image analysis of lettuce, mushrooms, and strawberries. The systems allowed to obtain the color parameters according to the CIE $L^*a^*b^*$ system and to obtain other important information as the size of the images for the calculation of the shrinkage index. Image size resulted to be an interesting outcome also for the correlation found with the weight of the samples, giving the possibility to use the parameter to estimate the weight loss during the storage without the use of a scale and reducing the manipulation of the sample. Considering the analysis conducted on foods during the treatment, ionization technology showed to not affect lettuce and mushroom, but in the case of strawberries, the exposition to NAI and low concentration of ozone lead to significant changes compared to the controls. Further investigation of the use of ionization technology on food should be considered. For example, the intermittent working mode could avoid a continuous exposition to oxidative species that cause into tissues excessive browning (mushroom) or deterioration (strawberry).

6 GENERAL DISCUSSION

6.1 Introduction

Nowadays, food quality and food waste are significant themes for consumers and represent worldwide social challenges. The food industry research has been continuously working to find suitable strategies to improve these aspects. Focusing on context consumption, in recent years people are increasingly demanding for consuming high-quality products also in their houses, but sometimes the foods they stored inside their refrigerators do not meet their requirements and were wasted. The decision of a consumer to eat a food strongly depends on the appearance of the food itself, therefore a potential solution could be to give the consumers the possibility to maintain as long as possible the high quality and fresh-like characteristics of the foods. This could be possible by the application of active air treatment technologies inside a domestic refrigerator. The development of a new generation of eco-friendly equipment can offer more prolonged storage of food thanks to better control of microbial growth and ensuring a better perception of food quality related to both freshness and safety.

The ambition of this Ph.D. thesis is to evaluate the possible application of ionization technology inside a domestic refrigerator to control microbial contamination of confined surfaces to maintain hygienic conditions and preserve the visual properties of foods for meeting the acceptability of the consumers.

6.2 Main findings

Part 1 investigated the antimicrobial effect of ionization technology on model systems. Food-related microorganisms were selected as targets to assess the efficacy of NAI

and ozone generated by the ionizers. In particular, the following results can be considered as the main findings:

- The amount of NAI and ozone produced depend on the device and are influenced by its intrinsic characteristics, like the electric stability during the working.
- Ionization technology affects the vitality of microorganisms. The antimicrobial activity is related to the amounts of NAI and ozone generated, to the microbial species exposed to the treatment, to the temperature, and to the exposure times. Find the right combination of these factors, allow to use of this technology in a safety application; for example, increase the exposure period to use devices that produce a lower amount of ozone.

Part 2 investigated the effect of ionization technology on food using vegetables, fruits, and mushrooms. The main findings are:

- Different concentrations of NAI and ozone lead to different levels of oxidative damages.
- The amount of ozone is considered a limiting factor for the application of the technology in a domestic refrigerator.

Part 3 investigated the ionization technology in a simulation of real application in a domestic refrigerator. glass. Polypropylene and polystyrene were used as representative materials for the antimicrobial assessment because they are used for the realization of inner lines of refrigerators. Lettuce, strawberry, and mushroom were selected as specified in the company's internal protocol for the evaluation of the food storage performances of the refrigerator drawers. The main findings are:

- The use of NAI with a low amount of ozone can achieve significant reductions in the microbial viability of food-related microorganisms. Moreover, *L. monocytogenes* results to be affected showing a reduction of 2 Log in all the tested materials.
- The treatment of lettuce, strawberry, and mushroom do not negatively influence the quality of treated food in terms of color change, weight loss, and

microbial load and then can be used inside domestic refrigerators to improve the microbiological quality of the surrounding environment.

6.3 Innovative aspects

The use of a color vision system was born to perform an accurate color determination analysis. This purpose was confirmed through the analysis of lettuce, strawberry, and mushroom during the ionization treatments. However, much information can be obtained by the photos taken with the vision machine. Weight loss during storage was correlated with the reduction of image size finding that a linear regression expressed the relationship between these two parameters. This finding gives the possibility to use image size to estimate the weight loss during the storage without the use of a scale and reducing the time of analysis and the manipulation of the sample. However, the preliminary study for deriving the equations must be done for each type of the investigated food.

6.4 Personal considerations

My Ph.D. was done in collaboration with Electrolux Italia S.p.A. This collaboration represents a situation in which the scientific approach is applied to an industrial application for the achievement of a common purpose. In this case, the aim was the improvement of the microbial quality of a refrigerator using green and low-cost strategies. The company suggested the application of ionization technology inside the drawers of domestic refrigerators, while the scientific approach aimed to evaluate the hygienic status of food, air, and surfaces and to assess the properties of food exposed to ionization underlining advantages and criticalities of the technology. Between the company and the university, my figure interposes to give continuous exchange of feedbacks to obtain the best possible result. I hope with my work to have made an important contribution for the possibility to place the ionizer inside a refrigerator, even if I am aware that more investigation should be done for this purpose. It would be nice to know to have contributed to the reduction of wasted food in the world.

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